Ligand binding and activation mechanism og the glucagon-like peptide-1 receptor

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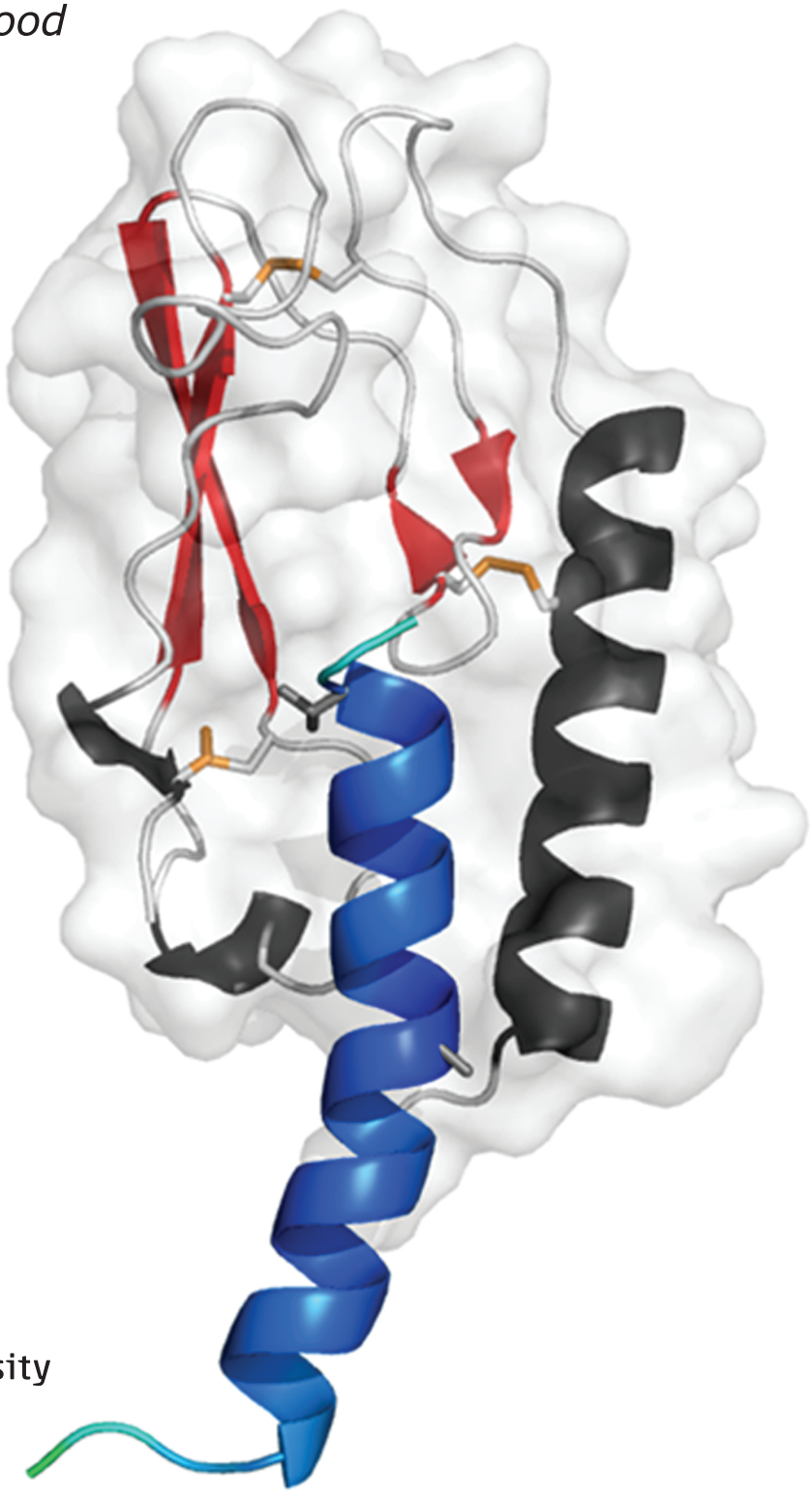

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PREFACE AND ACKNOWLEDGEMENTS

This thesis is submitted in partial fulfilment of the requirements for obtaining the Ph.D. degree at the Technical University of Denmark (DTU). The work was funded by a scholarship from Novo Nordisk A/S (2/3) and by a scholarship from DTU Chemistry (1/3).
The presented work was undertaken in the Department of GLP-1 and Obesity Biology (Novo Nordisk A/S) from June 2008 to December 2012. During this period, 18 months were spent on maternity leave. A small part of the work was carried out in the lab of Prof. Hans Bräuner-Osborne, Department of Drug Design and Pharmacology (University of Copenhagen) from April to August 2011. The work was supervised by Research Scientist, Ph.D. Steffen Reedtz-Runge (Novo Nordisk) and Associate Professor Günther H. Peters (Technical University of Denmark). Senior Principal Scientist Lotte Bjerre Knudsen and Project Director Patrick W. Garibay (Novo Nordisk) co-supervised the work.

During my thesis work, I have had the opportunity and pleasure of working with many wonderful and talented people. First and foremost, I wish to express my sincere gratitude to my main supervisor Steffen Reedtz-Runge for always being helpful, supportive and encouraging and for all his invaluable comments when discussing data. I would also like to thank Günther Peters for choosing to support the project financially, and for his helpful interaction throughout the work. In addition, great thanks go to Lotte Bjerre Knudsen and Patrick Garibay for their inspiring input to the work.
I have had the pleasure of working with several skilful technicians, but Karin H. Albrechtsen, Helle Iversen, Thomas S. Harkes, Carsten S. Stenvang and Mette H. Bien deserve a special thanks for their excellent help and great hands-on knowledge. In addition, I wish to thank all my colleagues for providing a positive working atmosphere and in particular Ph.D. fellows Cathrine Laustrup Møller and Mette Ladefoged for all the great times we have had together.
A warm acknowledgment goes to Hans Bräuner-Osborne, Jesper M. Mathiesen and Line Vedel for introducing me to cAMP biosensors and for their help and guidance during my 5 months at the University of Copenhagen.
Last but not least, I must thank my family and friends for their continued encouragement. In particular, I wish to thank my aunt Helle for helping with the layout, my parents for babysitting in times of need and especially my husband Chris, whose never-ending support has made this possible.

Christina Rye Underwood, December 2012

TABLE OF CONTENTS

ABBREVIATIONS

7TM	Seven-transmembrane
AC	Adenylyl cyclase
ADA	American Diabetes Association
Bpa	Benzoyl phenylalanine
cAMP	Cyclic adenosine monophosphate
CGRP	Calcitonin gene-related peptide
CGRP-R	Calcitonin gene-related peptide receptor
CLR	Calcitonin receptor-like receptor
CNS	Central nervous system
CRF	Corticotrophin-releasing factor
CRFR1	Type-1 CRF receptor
DAG	Diacylglycerol
DPP-IV	Dipeptidyl peptidase IV
ECD	N-terminal extracellular domain
ECFP	Enhanced cyan fluorescent protein
ECL	Extracellular loop
Epac	Exchange protein directly activated by cAMP
ERK	Extracellular signal-regulated kinase
FFA	Free fatty acids
FRET	Fluorescence resonance energy transfer
GCGR	Glucagon receptor
GEF	Guanine nucleotide exchange factor
GIP	Glucose-dependent insulinotropic polypeptide
GIP-R	GIP receptor
GLP-1/2	Glucagon-like peptide-1/2
GLP-1/2R	Glucagon-like peptide-1/2 receptor
GPCR	G-protein coupled receptor
HEK	Human embryonic kidney
ICL	Intracellular loop
ICV	Intracerebroventricular
IL	Interleukin
IP_3	Inositol triphosphate
IV	Intravenous
LOCI	Luminescent Oxygen Channeling Immunoassay
MAP	Mitogen-activated protein
mCer	monomeric form of Cerulean
mCit	monomeric form of Citrine
MTS	Methanethiosulfonate
NEP	Neutral endopeptidase
NMR	Nuclear magnetic resonance
NTS	Nucleus tractus solitarius
PAC-1-R	Type-1 PACAP receptor
PACAP	Pituitary adenylate cyclase-activating polypeptide
PKA	Protein kinase A
PLC	Phospholipase C

PTH	Parathyroid hormone
PTH-1R	Type-1 parathyroid hormone receptor
PTHrP	Parathyroid hormone related peptide
SNP	Single nucleotide polymorphism
T2D	Type 2 diabetes
T4L	T4 lysozyme
TFE	Trifluoroethanol
TM	Transmembrane
TNF	Tumor necrosis factor
ValPyr	Valine pyrrolidide
VIP	Vasoactive intestinal polypeptide
YFP	Yellow fluorescent protein
β_1AR	β_1-adreneric receptor
β_2AR	β_2-adreneric receptor

SUMMARY

In recent years, G-protein coupled receptors (GPCRs) have become important drug targets, which makes elucidation of their molecular structure and functional domains increasingly important for designing new and better therapeutic agents. The Glucagon-Like Peptide-1 receptor (GLP-1R) is a GPCR. Its endogenous agonist, Glucagon-Like Peptide-1 (GLP-1), has a number of physiological effects that contribute to reducing blood sugar and body weight. Therefore, GLP-1R has become a promising target for the treatment of type 2 diabetes (T2D).

The overall purpose of the Ph.D. project has been to investigate how GLP-1R interacts with receptor agonists. The thesis includes four studies, which investigate different aspects of these interactions. The first study elucidates GLP-1 binding to the extracellular domain of GLP-1R (ECD) (Study I), whereas the second study identifies receptor domains important for small molecule-mediated activation of GLP-1R (Study II). A fully functional, cysteine-deprived and C-terminally truncated GLP-1R is developed and characterised in Study III**.** In Study IV, a cAMP biosensor is used to investigate the cAMP kinetics of GLP-1R upon stimulation with different receptor agonists.

Collectively, the work has contributed to a more detailed understanding of GLP-1R pharmacology in a number of ways. A crystal structure elucidated the molecular details of GLP-1 binding to the ECD of GLP-1R and supported the existence of different binding modes of GLP-1 and exendin-4. In addition, the work established that seven cysteine residues in GLP-1R and more than half of the C-terminal tail are not required for GLP-1 binding or function. Last but not least, site-directed mutagenesis identified receptor domains and specific residues involved in small molecule-mediated activation of GLP-1R.

DANSK RESUMÉ

En del af de lægemidler, der er blevet fremstillet de seneste år er rettede mod G-protein koblede receptorer. For at kunne designe nye og bedre lægemidler er det derfor vigtigt at klarlægge den molekylære struktur og de funktionelle domæner i G-protein koblede receptorer. Glucagon-Lignende Peptid-1 receptoren (GLP-1R) er en G-protein koblet receptor. Den endogene agonist, Glucagon-Lignende Peptid-1 (GLP-1), har en række fysiologiske effekter, der bidrager til at reducere blodsukkeret samt kropsvægten. GLP-1R er derfor blevet en vigtig brik i behandlingen af type 2 diabetes.

Det overordnede formål med Ph.D. projektet har været at undersøge hvordan GLP-1R interagerer med agonister. Afhandlingen indeholder fire studier, der hver især belyser forskellige aspekter af disse interaktioner. Det første studie viser, hvordan GLP-1 binder til det ekstracellulære domæne i GLP-1R (ECD) (Studie I), hvorimod det andet studie identificerer domæner i GLP-1R, der er vigtige for, at små molekyler kan aktivere receptoren (Studie II). En funktionel, cystein-fattig og C-terminalt forkortet GLP-1R bliver fremstillet og karakteriseret i Studie III. I Studie IV benyttes en cAMP biosensor til at undersøge GLP-1R's cAMP kinetik efter stimulation med forskellige agonister.

Arbejdet har på flere måder bidraget til en mere detaljeret forståelse af GLP-1R's farmakologi. En krystalstruktur viste de molekylære detaljer i GLP-1 binding til det ECD af GLP-1R og understøttede teorien om, at GLP-1 og exendin-4 binder til receptoren på forskellige måder. Arbejdet fastslog desuden, at syv cysteiner i GLP-1R og mere end halvdelen af C-terminalen ikke er nødvendig for, at GLP-1 kan binde til- og aktivere receptoren. Sidst men ikke mindst blev mutagenese brugt til at identificere domæner og specifikke aminosyrer i receptoren, der er vigtige for, at små molekyler kan aktivere GLP-1R.

LIST OF STUDIES

The thesis is based on four studies, which will be referred to by their Roman numbers in the text.

Study I **Crystal Structure of GLP-1 in Complex with the Extracellular Domain of the GLP-1 Receptor**
Underwood, C.R., Garibay, P., Knudsen, L. B., Hastrup, S., Peters, G. H., Rudolph, R., Reedtz-Runge, S., The Journal of Biological Chemistry, 2010, 258, 723-730.

Study II **Transmembrane α-helix 2 and 7 are Important for Small Molecule-Mediated Activation of the GLP-1 Receptor**
Underwood, C. R., Knudsen, S. M., Wulff, B. S., Bräuner-Osborne, H., Lau, J., Knudsen, L. B., Peters, G. H., Reedtz-Runge, S., Pharmacology, 2011, 88, 340-348.

Study III **Development of a Cysteine-deprived and C-terminally Truncated GLP-1 Receptor**
Underwood, C. R., Knudsen, L. B., Garibay, P., Peters, G. H., Reedtz-Runge, S. (will be submitted to 'Peptides')

Study IV **Real-time cAMP Profiles of High- and Low Potency GLP-1 Receptor Agonists**

In addition to the scientific work, this thesis contains a general introduction to diabetes and GLP-1, followed by a general introduction to the structure of GPCRs with special focus on family B receptors. The introduction is followed by the 'Results and Discussion' section, which contains the scientific work. The work included in Study I and II was published in 2010 and 2011, respectively, and Study III will be submitted to 'Peptides' in the near future. The two papers and the manuscript are included in the thesis[1], and are each preceded by a short introduction. Study IV contains unpublished data, but some of the results are included in a co-authored paper that is currently in preparation. Concluding remarks are made at the end of the thesis.

[1] The two published papers are reprinted in this thesis with permission from the publishers

GENERAL INTRODUCTION

Type 2 diabetes

Obesity and type 2 diabetes

Numerous studies confirm that the prevalence of obesity has increased dramatically over the last three decades. Obesity has become one of the largest health challenges of the 21st century, affecting more than 500 million people worldwide (1). Overweight and obesity are major risk factors for chronic diseases like T2D ((2-4)), cardiovascular diseases ((3;5)), hypertension ((3;6)) and certain forms of cancer ((7-9)), which cause millions of deaths every year. However, obesity is a complex condition, and not everyone develops metabolic complications like insulin resistance and decreased insulin secretion, which can lead to T2D. It appears that the distribution of body fat plays a greater role in obesity-related complications than the total amount of body fat as such (10-12). There is now general agreement that a large amount of abdominal fat, in particular visceral fat, increases the risk of T2D (10;11;13). Obese subjects tend to have enlarged adipocytes (14), and enlarged adipocytes are resistant to the antilipolytic effects of insulin (15), which leads to higher concentrations of circulating free fatty acids (FFA). Elevated concentrations of FFA in plasma can lead to lipotoxicity (16), which can induce insulin resistance in muscle (17;18) and liver (19) as well as stimulate gluconeogenesis (20). Enlarged adipocytes do not only release FFA, they also release inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin (IL) 1, IL-6 (21-23) and other adipose-related hormones like leptin and adiponectin (24). These signalling molecules are commonly known as "adipokines", and together with FFA, they affect lipid metabolism and insulin sensitivity (25;26). Insulin resistance is the first step towards T2D. To deal with the increased demand for insulin, the β-cell is forced to enhance the secretion of the hormone. This β-cell compensation is initially successful and normal glucose levels are restored (27). For obesity and insulin resistance to be associated with T2D, β-cells must be unable to fully compensate for the decreased insulin sensitivity (28). Genetic factors (29;30) and age (31) seem to play an important part in the pathogenesis of T2D, as β-cell compensation fails in subjects that have "susceptible" islets as opposed to "robust" islets. In these subjects, the increased demand for insulin leads to β-cells dysfunction (32;33), which over time may result in blood glucose concentrations higher than normal, but lower than diabetes thresholds (prediabetes)[2]. Not everyone with prediabetes progresses to T2D. According to the American Diabetes Association (ADA), 5-10% of people with prediabetes develop T2D every year and up to 70% will eventually develop T2D (34).

The incretin effect and its implication in type 2 diabetes

In recent years, so-called incretin-based therapies have become available for the treatment of T2D. The "incretin effect" refers to the observation that insulin secretion increases substantially in response to orally ingested glucose compared to intravenous (IV) glucose administration (35). Two main incretin hormones have been identified, namely GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) (36-38). GIP was first identified as an incretin in the 1970's (39), and 10 years later, GLP-1 was identified as a product of the preproglucagon gene. In addition to glucagon, the preproglucagon gene was found to encode two related peptides named GLP-1 and GLP-2 (40). The two peptides were tested for their

[2] For a current definition of prediabetes, see (34).

ability to stimulate the release of insulin, but only GLP-1 was found to have insulinotropic activity (41). Human studies indicate that the overall incretin effect is reduced in people with T2D. This appears to be a result of decreased sensitivity to GIP, as GLP-1 but not GIP retains most of its insulinotropic activity in people with mild T2D (42).

Synthesis and degradation of GLP-1

A single gene encodes proglucagon in mammals, but tissue-specific posttranslational processing yields different peptides (43) (Figure 1). Proglucagon is processed by prohormone convertase PC1 into glicentin, oxyntomodulin, GLP-1 and GLP-2 in intestinal L-cells (44;45) (Figure 1). The full-length forms of GLP-1 (GLP-1(1-37)-OH and GLP-1(1-36)amide) are N-terminally truncated in L-cells, which yields two equally active isoforms, GLP-1(7-37)-OH and GLP-1(7-36)amide (43;46). Small amounts of full-length GLP-1(1-36)amide have also been detected in human pancreatic α-cells (47), where proglucagon is predominantly processed to glucagon (43;48) (Figure 1). GLP-1 is also produced in nucleus tractus solitarius (NTS) neurons in the brainstem (49;50).

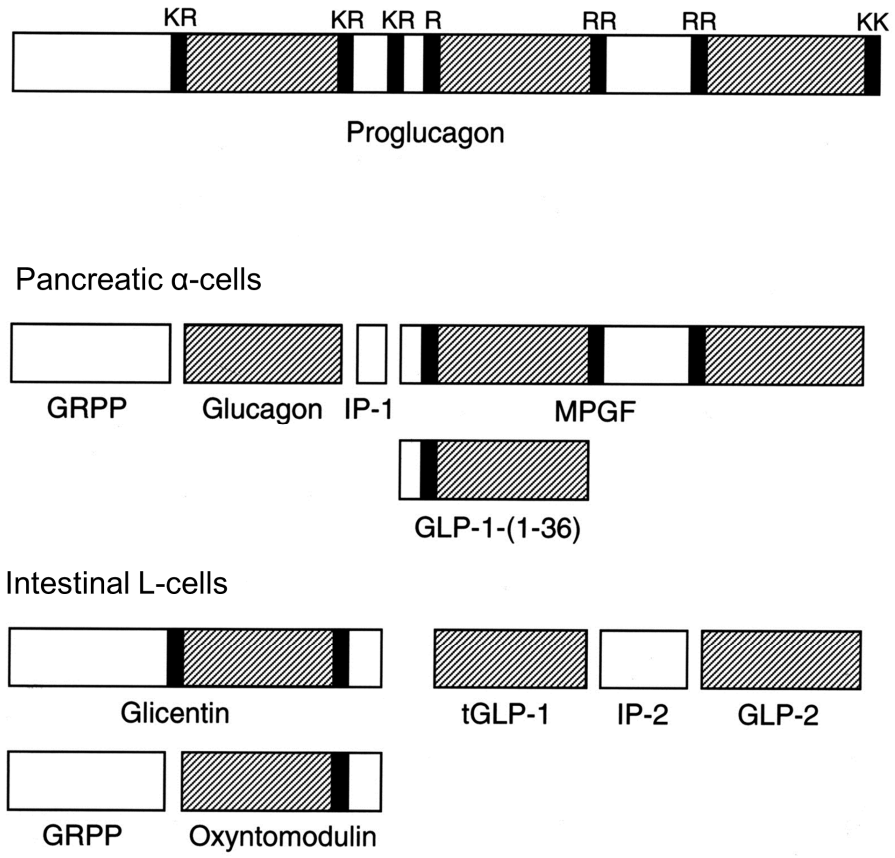

Figure 1. Schematic presentation of the structure and alternative processing of proglucagon. The basic amino acids lysine (K) and arginine (R) are sites for enzymatic cleavage. Proglucagon is cleaved by prohormone convertases in α-cells in the pancreas and in L-cells in the small intestine. The major bioactive peptides formed by cleavage are shaded. tGLP-1: Truncated GLP-1. The figure is reprinted with permission from the publisher (51).

The majority of L-cells are located in the distal part of the small intestine, yet, plasma levels of GLP-1 increase rapidly after food intake (52;53). In line with the role of GLP-1 as an incretin, oral ingestion but not IV administration of glucose increases the release of GLP-1 (53), and recently the same has been shown for oral ingestion versus IV administration of lipids (54).

The rapid secretion of GLP-1 after food intake has encouraged the idea that nutrient ingestion alone does not stimulate the release of GLP-1. It has been shown in several studies that the vagus nerve may play an important role in the secretion of GLP-1 from L-cells (55;56). After the initial nutrient-stimulated rise in circulating levels of GLP-1, levels fall rapidly due to renal clearance and N-terminal degradation of the peptide by dipeptidyl peptidase IV (DPP-IV) (57-59). DPP-IV-mediated cleavage of GLP-1 results in the formation of GLP-1(9-36)amide and GLP-1(9-37)-OH (57;60). The biological function of GLP-1(9-36)amide has been discussed over the years and research is still on-going. A few studies have suggested that GLP-1(9-36)amide is an antagonist of GLP-1R (61;62), whereas other studies have suggested that the metabolite acts as a weak agonist of GLP-1R (63). However, it is important to notice that GLP-1(9-36)amide has no effect on insulin secretion (64). The short half-life of native GLP-1 limits its therapeutic potential, so various strategies have been employed to extend the *in vivo* half-life of this hormone.

The therapeutic potential of GLP-1 receptor agonists

Two GLP-1R agonists, exenatide and liraglutide, are marketed for treatment of T2D. Exenatide is a synthetic version of exendin-4, which is a peptide isolated from the venom of the lizard *Heloderma suspectum* (65). GLP-1 and exendin-4 are approximately 50% identical, and exendin-4 binds to- and activates GLP-1R with similar affinity and potency compared to GLP-1 (66). The human half-life of exenatide is 2.4 hours (67) compared to 2-3 minutes for native GLP-1, which necessitates two daily injections of this drug. Liraglutide is an acylated albumin-bound human GLP-1 analogue with a human half-life of 11-13 hours, which makes this drug suitable for once-daily administration (68). Hence, liraglutide and exenatide may be classified as long-acting and short-acting GLP-1R agonists, respectively (69). Both GLP-1 analogues possess the beneficial effects of native GLP-1 (Figure 2), although they may result in side effects such as nausea and diarrhea. However, these adverse effects are typically mild and subside after 4-8 weeks of treatment (70-74).

Physiological and pharmacological effects of GLP-1R agonists

The first studies of the physiological role of GLP-1 were performed in the mid-1980s, and used the full-length forms of GLP-1. The full-length peptides showed no biological activity, and a few years later, it was discovered that the full-length isoforms were N-terminally truncated (43;75). Most of the circulating GLP-1 is found in the GLP-1(7-36)amide form, but small amounts of GLP-1(7-37)-OH are also detectable (76). Today, it is evident that GLP-1 has numerous physiological effects (Figure 2).

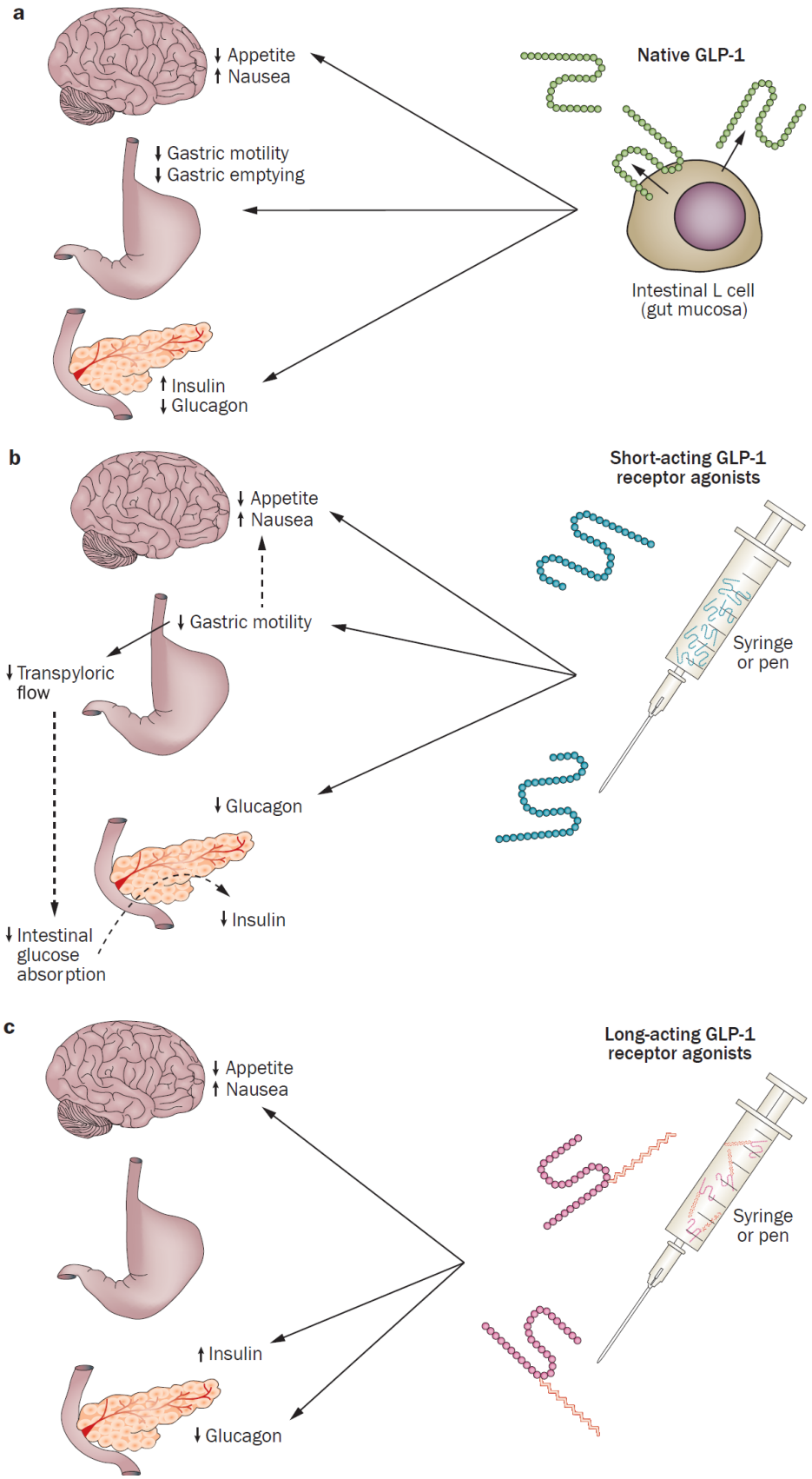

Figure 2. Overview of the physiological and pharmacological actions of GLP-1R agonists. Most of the physiological actions of native GLP-1 **(A)** are also observed for short-acting GLP-1R agonists like exenatide **(B)** and long-acting GLP-1R agonists like liraglutide **(C)**. The figure is reprinted with permission from the publisher (69).

The effect of GLP-1R agonists on blood glucose levels

The first discovered physiological function of GLP-1 is insulin secretion from pancreatic β-cells, where GLP-1(7-37)-OH and GLP-1(7-36)amide are equally potent in stimulating glucose-dependent insulin secretion (77;78). The insulinotropic action of GLP-1 on β-cells is attenuated when glucose levels fall, hence GLP-1 cannot induce severe hypoglycaemia. Not only does GLP-1 potentiate insulin secretion from β-cells, it also stimulates transcription of the proinsulin gene (79). In addition, GLP-1 inhibits the secretion of glucagon from pancreatic α-cells (80), which is probably mediated through the local release of somatostatin from δ-cells in the pancreas (80;81). In the fasting state, the insulin-stimulating- and glucagon-suppressing actions of GLP-1 appear to contribute almost equally to the reduction of blood glucose levels (82). Conversely, the delaying effect of GLP-1 on gastric emptying seems to account almost entirely for its postprandial control of blood glucose (83;84). GLP-1 inhibits gastric emptying (85;86), which in turn slows the entry of nutrients such as glucose into the circulation (84).

GLP-1 analogues like liraglutide and exenatide also reduce blood glucose levels, but their modes of action differ due to their different pharmacokinetic profiles (Figure 2). Short-acting GLP-1R agonists like exenatide primarily reduce postprandial blood glucose levels by inhibiting gastric emptying (67;87), but these GLP-1 analogous have limited effect on fasting glucose levels (70;88). Long-acting GLP-1R agonists like liraglutide show no significant delay of gastric emptying (89;90), and as a consequence, these GLP-1 analogues have limited effect on postprandial glucose control (70). However, the long-acting GLP-1R agonists have a stronger effect on fasting glucose levels compared to their short-acting counterparts (70;88). Both short- and long-acting GLP-1R agonists reduce the secretion of glucagon from pancreatic α-cells (69).

The effect of GLP-1R agonists on body weight

As mentioned, obesity is tightly linked to the development of T2D. GLP-1 has been shown to reduce body weight in numerous of studies, but the exact mechanism by which GLP-1 influences food intake (and weight) is not fully understood. GLP-1 inhibits gastric emptying (85;86), which stimulates satiety. However, long-acting GLP-1R agonists cause a reduction in body weight that is comparable to that observed for short-acting GLP-1R agonists (70;88) even though the long-acting compounds have no significant effect on gastric emptying (89;90). Hence, the weight-reducing effect of native GLP-1 and GLP-1 analogues appears to be independent of gastric emptying (90), but may be a result of their actions in the central nervous system (CNS). One of the first studies that demonstrated GLP-1 action in CNS showed that intracerebroventricular (ICV) administration of GLP-1 decreased food intake in fasted rats. ICV injection of the specific GLP-1R antagonist, exendin-4(9-39), blocked the inhibitory effect of GLP-1 on food intake, indicating that the appetite-suppressing effect of ICV injected GLP-1 is mediated by specific interactions with GLP-1Rs in the brain (91). Several studies have now demonstrated that multiple brain regions could be involved in transducing the appetite-suppressing effect of GLP-1 (92-94).

Additional biological effects of GLP-1R agonists

Cardiovascular disease is one of the long-term complications of obesity and T2D. High affinity receptors for GLP-1 have been located in the heart (95;96), and administration of GLP-1 has been shown to improve cardiovascular functions. For example, GLP-1 has been shown to improve endothelial function in T2D patients with coronary heart disease (97), and GLP-1 also appears to improve left ventricular dysfunction in patients suffering from heart failure (98). In

addition, native GLP-1 as well as GLP-1 analogues have the beneficial effect of reducing systolic blood pressure (70-74). However, long-acting GLP-1R agonists also increase the heart rate by 2-5 beats per minute (bpm) (70;99), although the underlying mechanism is not yet fully understood. The augmented heart rate does not appear to cause increased incidents of cardiovascular events (100).
In addition to the positive cardiovascular effects, GLP-1 has also shown promising proliferating effects on β-cells and neurons. Declining β-cell function is one of the hallmarks of T2D, and antidiabetic treatment often requires intensification as the disease progresses (101). Animal and *in vitro* studies indicate that GLP-1 increases β-cell mass through a combination of increased β-cell neogenesis and proliferation as well as reduced apoptosis (102-104). More importantly, GLP-1 appears to improve β-cell function in patients with T2D (105;106). The prevalence of Alzheimer's disease is also increased in patients with T2D (107;108). GLP-1 appears to have neuroprotective effects in CNS (109) as well as stimulate neurogenesis (110). Hence, GLP-1 may also turn out to be useful in the treatment of neurodegenerative diseases.

G-protein coupled receptors

General introduction to GPCRs

The superfamily of GPCRs, also known as seven-transmembrane (7TM) receptors, constitutes one of the largest families of proteins in the human genome (111). GPCRs consist of an extracellular N-terminus, seven membrane-spanning α-helices connected by three extracellular and three intracellular loops, and an intracellular C-terminus (112). It is believed that all GPCRs share the same characteristic topology, as the receptors interact with a common intracellular repertoire of heterotrimeric GTP-binding proteins (G-proteins) (Figure 3). The structural variety among GPCRs is related primarily to a great diversity of ligands (ions, nucleosides, peptide hormones, glycoproteins, amino acids etc.). Ligand binding to a GPCR leads to conformational changes in the interior part of the TM domain, resulting in activation of specific G-proteins, which in turn activate downstream effector proteins such as adenylyl cyclase (AC), phospholipase C (PLC) and ion channels (Figure 3) (113). However, many GPCRs also exhibit more complex signalling behaviour like constitutive activity (114), biased agonism/functional selectivity (115;116) and G-protein-independent signalling (115;117).

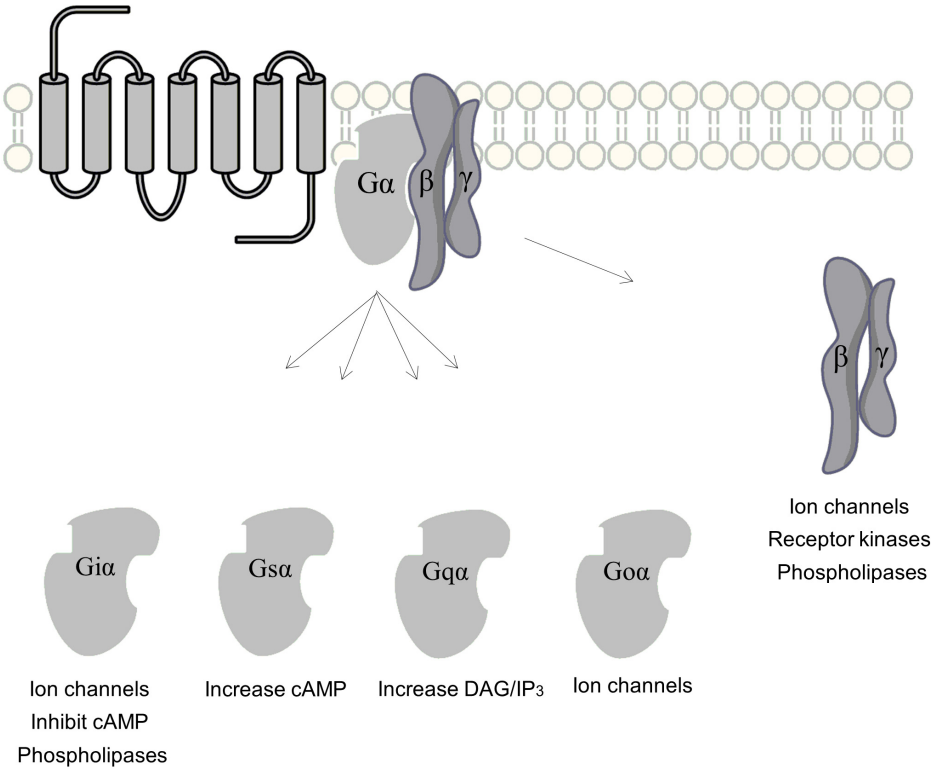

Figure 3. Schematic representation of GPCR signal transduction pathways. Agonist binding to a GPCR leads to conformational changes in the intracellular part of the TM domain, which enables binding of a heterotrimeric G-protein (α, β and γ subunit). GDP is exchanged for GTP in the α-subunit upon formation of the GPCR-G-protein complex, which leads to dissociation of the α-subunit from the βγ heterodimer. Depending on the isoform, the α-subunit as well as the βγ heterodimer regulate various effector proteins (113). cAMP: Cyclic adenosine monophosphate, DAG: Diacylglycerol, IP_3: Inositol triphosphate.

Family A GPCRs

Based on protein sequence similarity, GPCRs can be divided into five families: Rhodopsin (family A), secretin (family B), glutamate (family C), adhesion and Frizzled/Taste 2 receptors (112). Family A is by far the largest and best characterised GPCR family. More than 85% of the human GPCRs are thought to belong to family A (112), which makes this family particularly interesting in terms of drug discovery and development. A prerequisite for designing new and improved drugs is to understand how different ligands interact with GPCRs and how these ligands control the equilibrium between active and inactive conformational states. X-ray crystallography is currently the most favoured technique for structural determination of GPCRs, as it can provide detailed information about specific receptor-ligand interactions and the conformational changes associated with GPCR activation. Hence, structural determination of GPCRs, in particular family A GPCRs, has progressed rapidly over the past 10 years and provided invaluable information about the molecular details of ligand binding and receptor activation.

Until recently, the only known structure of a GPCR was that of bovine rhodopsin covalently bound to the chromophore 11-*cis*-retinal, which represents the 'dark' inactive state of the molecule (118). The crystal structure of rhodopsin completed decades of biochemical and biophysical work on the interrelationship of the seven α-helices in GPCRs (119-121). However, although representing family A GPCRs, bovine rhodopsin shows little sequence homology with other family A receptors. The first crystal structures of a typical family A GPCR were published

recently: The β_2-adreneric receptor (β_2AR) bound to the inverse agonist carazolol (122-124). These structures were soon succeeded by crystal structures of other ligand-activated family A GPCRs, all of which represented inactive conformations of the receptor in question (125;126). Recently, crystal structures of a thermostabilised avian β_1-adreneric receptor (β_1AR) coupled to two full agonists were published (127). However, the structures represented inactive states of the receptor, possibly due to six thermostabilising mutations that restricted the receptor in an inactive conformation. Simultaneously, a different research group published two crystal structures of agonist-bound β_2AR, of which one represented an active conformation (128;129). It became clear that agonist binding in itself is not enough to stabilise β_2AR in an active conformation. Stabilisation of the active state requires simultaneous binding of agonist and G_s, the stimulatory G-protein for AC (128). Shortly after the release of the agonist-bound structures, a crystal structure of β_2AR in complex with a nucleotide-free G_s-protein was published (130).

Inactive versus active conformation of β_2AR

The crystal structures of β_2AR in complex with the inverse agonist carazolol (122-124) were the first GPCR structures published since those of rhodopsin. Previous fluorescence resonance energy transfer (FRET) based studies had shown that the C-terminal and the third intracellular loop (ICL3) were the most unstructured regions of β_2AR (131). The last 48 amino acids were removed from the C-terminal of β_2AR (122;123), and two different approaches were employed to stabilise ICL3. The first approach used a monoclonal antibody (Mab5) that bound to ICL3 (123). This stabilised the receptor enough to facilitate crystallisation, but the binding pocket was poorly resolved in the 3.4 Å resolution structure (123). The second approach replaced most of ICL3 with T4 lysozyme (T4L), which stabilised the receptor and resulted in a 2.4 Å resolution structure (122).

The overall structure of β_2AR is similar to that of rhodopsin with seven TM helices organised in a counter clockwise orientation as seen from the extracellular side of the membrane (Figure 4A). In addition, an eighth helix resides parallel to the cytoplasmic face of the membrane. A similar helix is believed to exist in all family A GPCRs (132) and most likely also in some family B GPCRs (133;134). Transmembrane helix 2 (TM2), TM5, TM6 and TM7 are bent around Pro residues. Helix kinks induced by Pro residues are also observed in rhodopsin, and are thought to facilitate the structural changes involved in receptor activation (135). The second extracellular loop (ECL2) of β_2AR contains a short α-helical segment, which is not observed in rhodopsin (Figure 4B). The rigid structure of ECL2 exposes the binding pocket of β_2AR to solvent, despite the fact that it is buried between TM3, TM4, TM5 and TM7 (122).

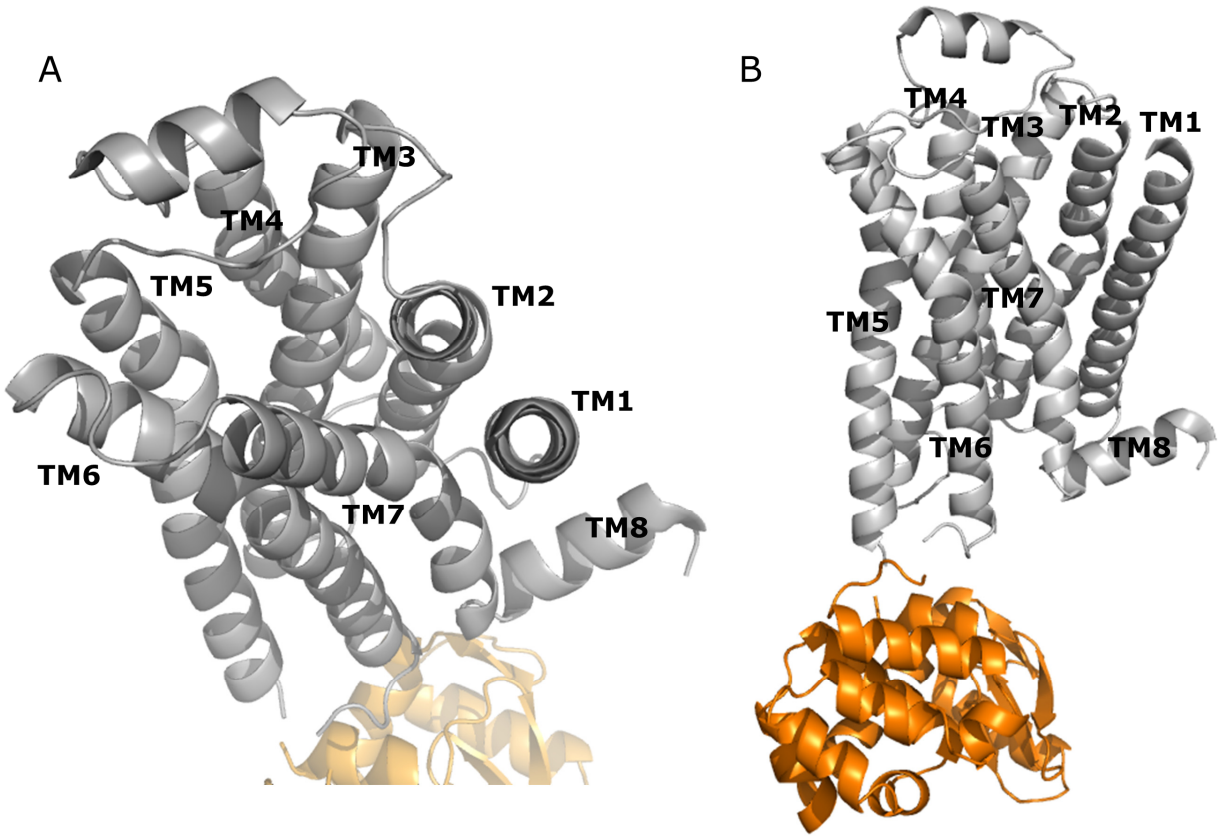

Figure 4. Structure of the β_2AR-T4L fusion protein (inactive β_2AR structure). The receptor and T4L are coloured in grey and orange, respectively. **(A)** The overall structure of β_2AR-T4L as viewed from the extracellular side of the plasmamembrane. **(B)** β_2AR-T4L as viewed from the side. T4L is fused between the cytoplasmic ends of TM5 and TM6, but in the crystal structure, T4L is tilted away from the receptor, resulting in limited contact between the two proteins (PDB ID: 2RH1).

In an effort to crystallise the active conformation of β_2AR, researchers identified a high affinity, slow off-rate agonist, BI-167107 (128). Two different approaches were employed to stabilise an active conformation of the receptor. Initially, the small antibody (nanobody) Nb80 was used as a surrogate for the G_s-protein, and the final structure contained BI-167107-bound β_2AR-T4L in complex with Nb80 (128). Shortly after, BI-167107-bound β_2AR-T4L was crystallised in complex with a nucleotide-free G_s-protein (130). Instead of replacing ICL3, T4L was now fused to the unstructured N-terminal domain of β_2AR, and the complex was further stabilised with a nanobody (Nb35) bound to the $G_{\alpha s}$-subunit. The overall structure of β_2AR is very similar in the Nb80- and the G_s-coupled state (overall root mean square deviation ~ 0.6Å). More importantly, the two structures are very similar near the highly conserved E/DRY motif in the cytoplasmic end of TM3. The E/DRY sequence is found in ~70% of all family A GPCRs (124), and is thought to be important for maintaining the receptor in an inactive conformation (136). In the inactive state of rhodopsin, Glu^{134} and Arg^{135} of the ERY motif form hydrogen bonds and ionic interactions with Glu^{247} in the cytoplasmic end of TM6. This has been referred to as the "ionic lock", as the lock is broken upon activation of rhodopsin (136). However, the two crystal structures of carazolol-bound β_2AR agree that the ionic lock appears to be broken in the inactive state of β_2AR (124), as Asp^{130} and Arg^{131} of the DRY motif interact primarily with each other rather than with Glu^{268} in TM6. In addition, the distance between TM3 and TM6 is greater than observed for rhodopsin, indicating that the ionic lock is not essential for maintaining an

inactive conformation of β_2AR in the crystal. Comparison of the agonist- and carazolol-bound structures of β_2AR reveals that the cytoplasmic ends of TM5 and TM6 move outwards upon agonist binding (130) (Figure 5A), which resembles the conformational changes observed for activation of rhodopsin (136). In addition, TM5 is extended with seven residues in the cytoplasmic end (Figure 5A).

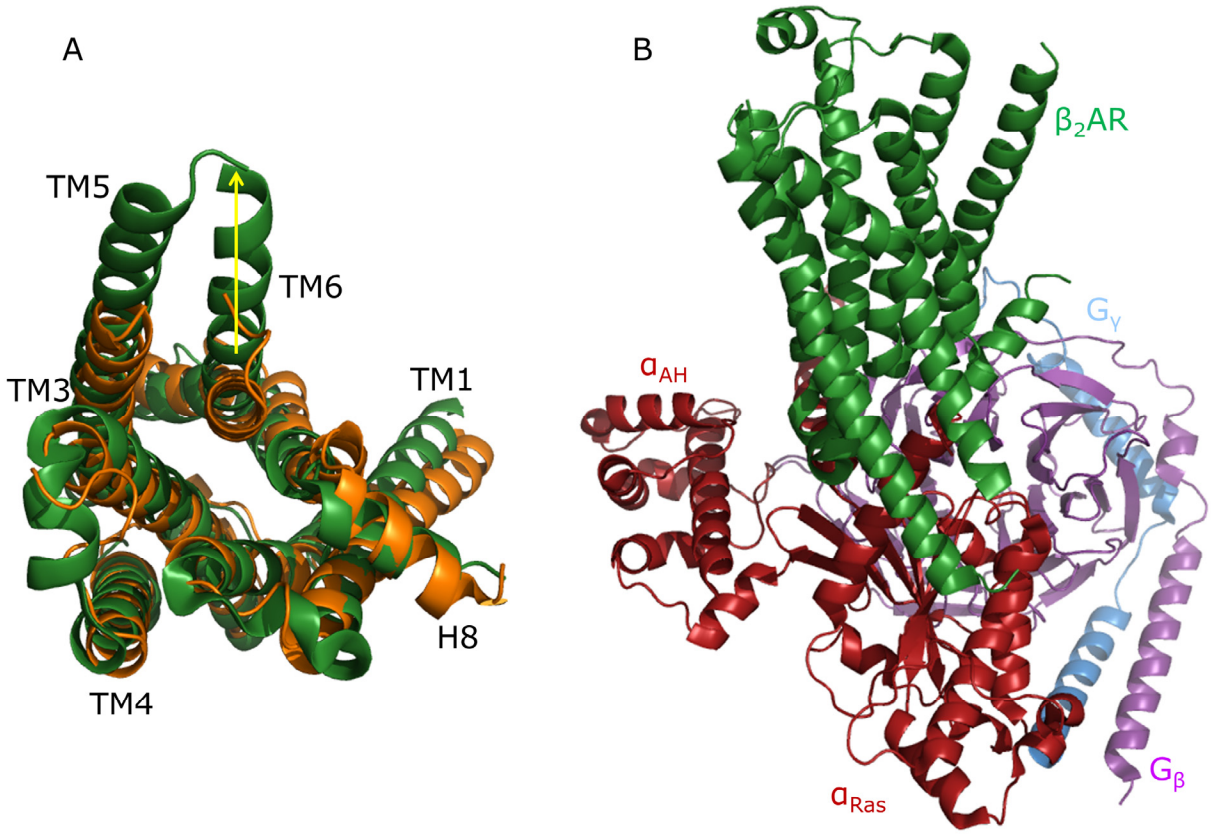

Figure 5. Conformational changes associated with agonist binding to β_2AR. (A) Superposition of the β_2AR-G_s structure (green, PDB ID: 3SN6) and the carazolol-bound β_2AR structure (orange, PDB ID: 2RH1) as viewed from the cytoplasmic side of the plasmamembrane. The main rearrangements involve a 14 Å outward movement of TM6, as indicated with a yellow arrow, and an extension of TM5 by two helical turns (130). **(B)** Structure of the nucleotide-free β_2AR-G_s complex (PDB ID: 3SN6)**.** $G_{\alpha s}$ (red) is made up of two subdomains; a GTPase domain that interacts with β_2AR and the β-subunit (α_{Ras}), and an α-helical domain (α_{AH}). The interface between the two domains forms the nucleotide-binding pocket, but in the nucleotide-free state, the α_{AH} domain has a variable position relative to the rest of the complex (130).

The crystal structure of G_s-coupled β_2AR has for the first time uncovered the structure of an activated G_s-protein. G_s consists of three subunits, α, β and γ (Figure 5B). $G_{\alpha s}$ is made up of two subdomains, a GTPase domain that interacts with β_2AR and the β-subunit (α_{Ras}), and an α-helical domain (α_{AH}). The interface between the two domains forms the nucleotide-binding pocket (130) (Figure 5B). In the agonist-bound state, β_2AR does not interact with the β- or the γ-subunit of G_s. Instead, the active conformation of β_2AR is stabilised by interactions between the α_{Ras} domain of G_s and ICL2, TM5, and TM6 of β_2AR. Sequence alignment with other GPCRs indicates that there is no conserved motif for G_s-coupling. Only one amino acid, Phe^{139} in ICL2, which is partially conserved in other G_s-coupled receptors, interacts with $G_{\alpha s}$.

Family B GPCRs

Family B of the GPCR superfamily was initially defined in 1991 by the cloning and expression of the rat secretin receptor (137). Subsequently, other family B receptors were cloned including the parathyroid hormone (PTH) receptor (138), the calcitonin receptor (139), the GIP receptor (GIP-R) (140), the corticotrophin-releasing factor (CRF) receptor (141), the vasoactive intestinal polypeptide (VIP) receptor (142), the pituitary adenylate cyclase-activating polypeptide (PACAP) type I receptor (143) and the glucagon receptor (GCGR) (144). The GCGR branch consists of GCGR, GLP-1R, GLP-2R and GIP-R.

Family B GPCRs are characterised by a relatively large ECD of about 120-150 amino acid residues. The ECD is essential for peptide ligand binding, and the structural integrity of the ECD relies on three conserved disulphide bonds and several conserved residues, which will be described in detail in the following paragraph. The first crystal structure of a TM domain of a family B GPCR was solved recently. The N- and C-termini were deleted from the type-1 CRF receptor (CRFR1), and several thermostabilising mutations were introduced. These modifications facilitated crystallisation of the receptor, and the structure was solved to 2.6 Å resolution (145). As of December 2012, limited information is available on the structure itself, but it has been shown that the extracellular part of the TM domain adopts a much more open, V-shaped conformation compared to family A GPCRs. The open conformation of CRFR1 probably represents the general structure of family B GPCRs, as this family of receptors binds large peptide ligands and have a large ECD. The intracellular part of the TM domain of CRFR1 is strikingly similar to the intracellular part of family A GPCRs (145). This is not surprising as GPCRs of different families activate similar G-proteins.

The crystal structure of CRFR1 most likely represents the antagonist-bound (inactive) state, and does not provide any information about peptide binding to the receptor. It is assumed that peptide ligands bind to family B GPCRs according to the two-domain model. This model suggests that the C-terminal part of the peptide binds the ECD of the receptor while the N-terminal part of the peptide binds to the TM domain and extracellular loops (Figure 6). Ligand binding induces a conformational change that enables the intracellular segments of the receptor to trigger signalling events (reviewed in (146)).

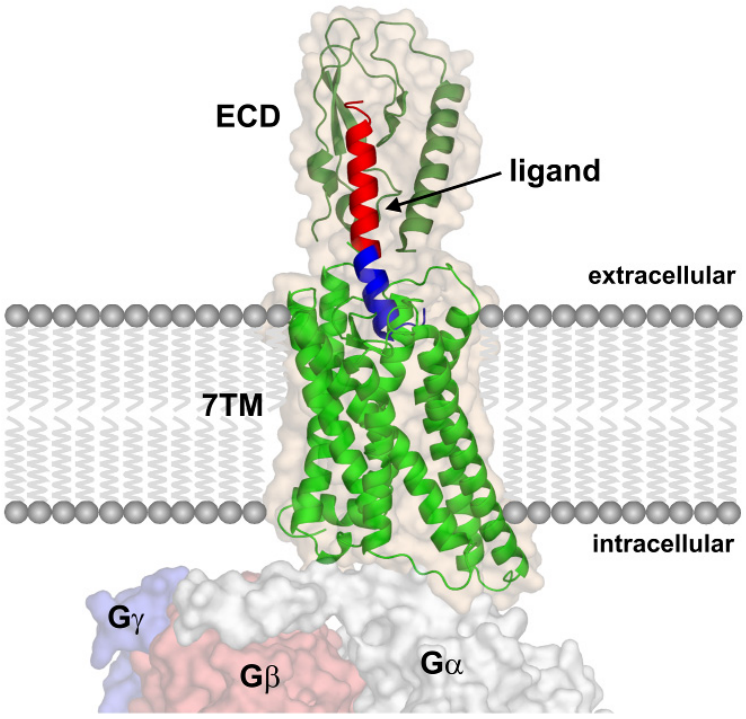

Figure 6. A model for ligand binding to family B GPCRs. Peptide ligands are believed to bind to family B GPCRs according to the two-domain model. First, the C-terminal part of the ligand (red) binds the ECD of the receptor (dark green), followed by binding of the N-terminal part of the ligand (blue) to the TM receptor domain (light green). Structural rearrangements in the receptor activate the G_s-protein, leading to dissociation of the Gα subunit and formation of the second messenger cAMP. The figure is reprinted with permission from the publisher (147).

The binding orientation of family B GPCR ligands was first acknowledged in studies of chimeric peptides and receptors (148-153). The two-domain model is also generally consistent with photoaffinity cross-linking studies of several family B GPCRs. Photoreactive side chains in the C-terminal part of the peptide ligand interact with residues in the ECD of the receptor, whereas photoreactive side chains in the N-terminal part of the ligand interact with the TM receptor domain upon exposure to UV-light (154-157). Most recently, substantial evidence for the two-domain model has come from the structural characterisation of the isolated ECDs of several family B GPCRs. The first structure of an isolated ECD of a family B GPCR was solved by nuclear magnetic resonance (NMR) spectroscopy (158). Subsequently, structures of ligand-bound ECDs were solved including the ECD of CRFR1 (159), the type-1 PACAP receptor (PAC1-R) (160), GIP-R (161), GLP-1R (162) and the type-1 PTH receptor (PTH-1R) (163). ECDs of family B receptors all share a common molecular architecture, termed the 'secretin family recognition fold' (164), which is described in detail in the following section.

The crystal structure of CRFR1 is devoid of an ECD, so the orientation of the ECD relative to the TM domain is still unknown for family B GPCRs. However, a recent study identified blocking antibodies that bind to the ECD of GCGR (165). A combination of chimeric receptors and site-directed mutagenesis led the authors to suggest that the ECD of GCGR may interact with ECL3 in the absence of an agonist, which predominantly retains GCGR in an inactive conformation. Glucagon binding may uncouple the ECD from ECL3 and in this way stabilise an active receptor conformation (165). This inhibitory effect of the ECD has not (yet) been reported for other family B GPCRs. However, the ECD of GLP-1R has been implicated directly in receptor activation, as an endogenous agonist sequence, NRTFD, was proposed to exist in the ECD of GLP-1R (166). Interestingly, a photolabile benzoyl phenylalanine (Bpa) was recently introduced into the pentapeptide derived from this sequence. Photo-induced cross-linking

subsequently showed that NRTFD binds to ECL3 of GLP-1R (167), suggesting that the ECD and ECL3 may also be functionally coupled in GLP-1R.

The Glucagon-Like Peptide-1 Receptor

In the 1980's, it was suggested that GLP-1 acts through specific receptors located on the surface of pancreatic β-cells, as high-affinity binding sites for GLP-1 (K_d = 200 pM) and activation of cAMP signal transduction (EC_{50} = 250 pM) were identified in insulinoma β-cell lines (168). The rat GLP-1R was cloned in 1992 from a rat pancreatic islet cDNA library (169) followed by cloning of the human GLP-1R in 1993 (170). Today, GLP-1Rs have been detected in several tissues including pancreas, lung, GI-tract, kidney, heart and brain (95).
The GLP-1R consists of a predicted signal sequence, an ECD of approximately 120 residues, seven membrane-spanning α-helices connected by three extracellular and three intracellular loops (the TM domain), and a C-terminal intracellular tail. The ECD contains three disulphide bonds and three potential asparagine (N)-linked glycosylation sites (169). Inhibition of the glycosylation process with tunicamycin reduced the number of GLP-1 binding sites, indicating that glycosylation of GLP-1R ECD is important for receptor expression at the cell surface but not for ligand binding (171).

The N-terminal Extracellular Domain

The isolated ECD of GLP-1R can be expressed recombinantly in *E. coli* inclusion bodies and refolded to form a functional receptor domain (172-174). GLP-1 binds to the isolated ECD with reduced affinity (IC_{50} > 500 nM), compared to the full-length receptor (IC_{50} ~0.5 nM) (173;174), suggesting that additional binding determinants of GLP-1 are present in the TM domain in agreement with the two-domain binding model. Interestingly, exendin-4 and exendin-4(9-39) maintain high affinity for the isolated ECD (IC_{50} = 6 nM compared to IC_{50} ~0.5 nM for the full-length receptor) (173;174). The molecular details of GLP-1 binding to the ECD are described in Study I.
The core structure of GLP-1R ECD represents the typical 'secretin family recognition fold' of family B GPCRs (Figure 7A) with an N-terminal α-helix (residues Leu^{32}-Glu^{52}) linked by a disulphide bond to a central core consisting of two regions of antiparallel β-sheets (β-strand β_1-β_4). Residues Thr^{65}-Phe^{66} (β_1) and Cys^{71}-Trp^{72} (β_2) constitute the first region of antiparallel β-sheets, and the second region is comprised of residues Gly^{78}-Ser^{84} (β_3) and His^{99}-Thr^{105} (β_4) (Figure 7A and B) (162). Six loop regions (L_1-L_6) are present between the α-helix and β-strands. The tertiary structure is stabilised by three conserved disulphide bonds and by several intramolecular interactions (162). The α-helix is connected to β_2 through a disulphide bond between Cys^{46} and Cys^{71} (Figure 8A). The α-helix also interacts with β_1, as the side chain of Arg^{64} (β_1) forms hydrogen bonds with the backbone of Leu^{50}, Asp^{53} and Pro^{54} (Figure 8A). The two regions of antiparallel β-sheets are joined by the disulphide bond between Cys^{62} (before β_1) and Cys^{104} (β_4). The last disulphide bond connects the end of β_3 (Cys^{85}) to a small α-helical segment in the C-terminal part of the ECD (Cys^{126}) (162).
In addition to the three disulphide bonds, six residues are conserved in the ECD of all family B GPCRs (Asp^{67}, Trp^{72}, Pro^{86}, Arg^{102}, Gly^{108} and Trp^{110} in GLP-1R) and they are important for the tertiary structure (162) (Figure 8B). The carboxyl group of Asp^{67} stabilises the turn between β_1 and β_2 through hydrogen bonds with the backbone nitrogens of Tyr^{69} and Ala^{70}. In addition, the carboxyl group of Asp^{67} forms hydrogen bonds with the side chain nitrogens of Arg^{102} on β_4 and Trp^{72} on β_2. Arg^{102} is aligned between the side chains of Trp^{72} and Trp^{110} forming a central cluster, which is also observed in other structures of family B ECDs (158;161).

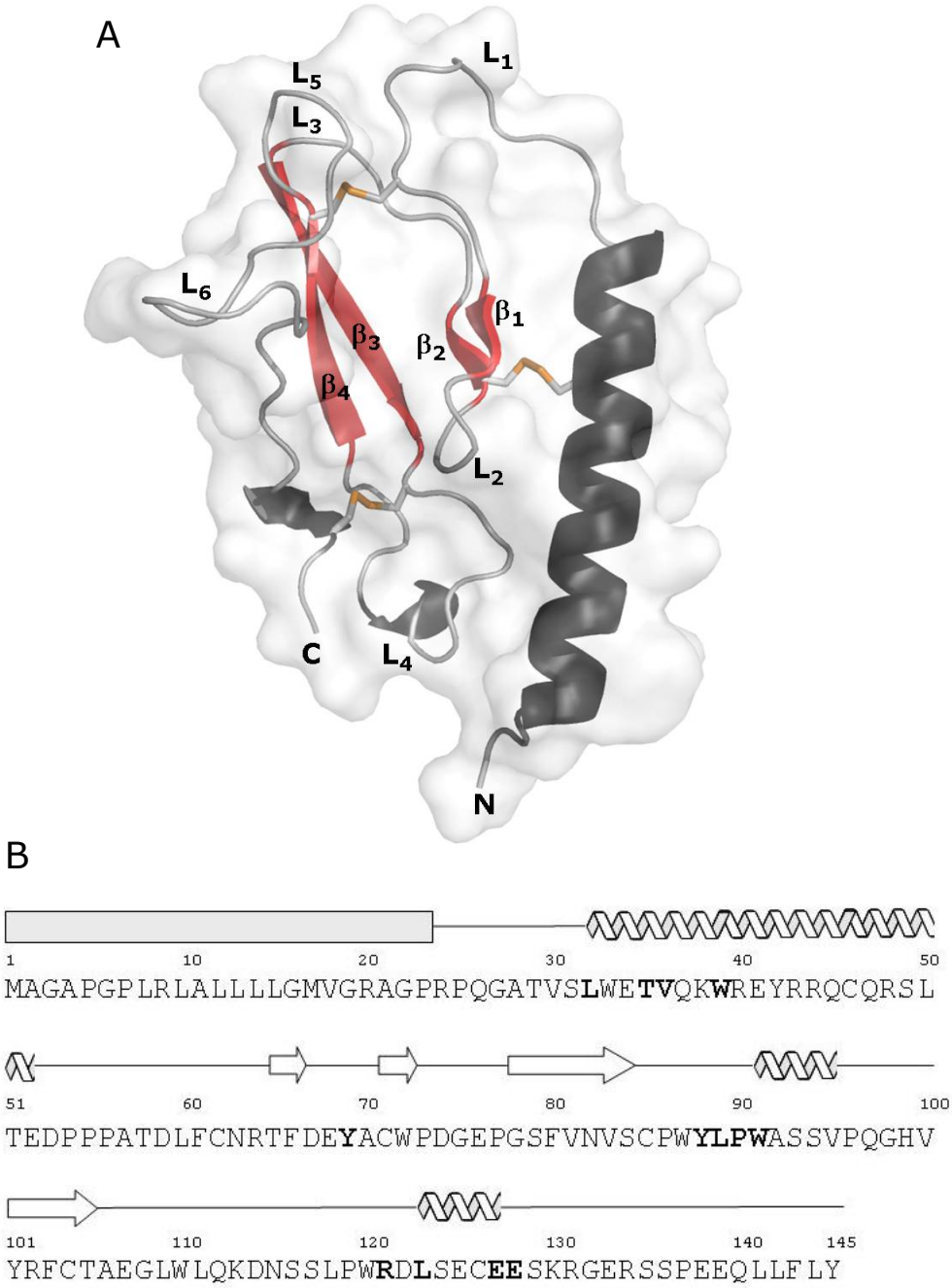

Figure 7. The ECD of GLP-1R. (A) Ribbon representation of the structure of GLP-1R ECD (α-helix in black, β-strands in red and loops in grey). Disulphide bonds are shown as orange sticks, and loop regions L_1-L_6 are indicated. Residues Thr^{65}-Phe^{66} (β_1) and Cys^{71}-Trp^{72} (β_2) constitute the first region of antiparallel β-sheets, and the second region is comprised of residues Gly^{78}-Ser^{84} (β_3) and His^{99}-Thr^{105} (β_4). **(B)** Sequence of GLP-1R ECD (residues 1-145). Residues shown in bold form the ligand binding site, and the secondary structure is depicted in the panel above the sequence. The predicted signal sequence is indicated by a grey rectangle, α-helical segments are shown as helixes and β-strands are shown as arrows (147).

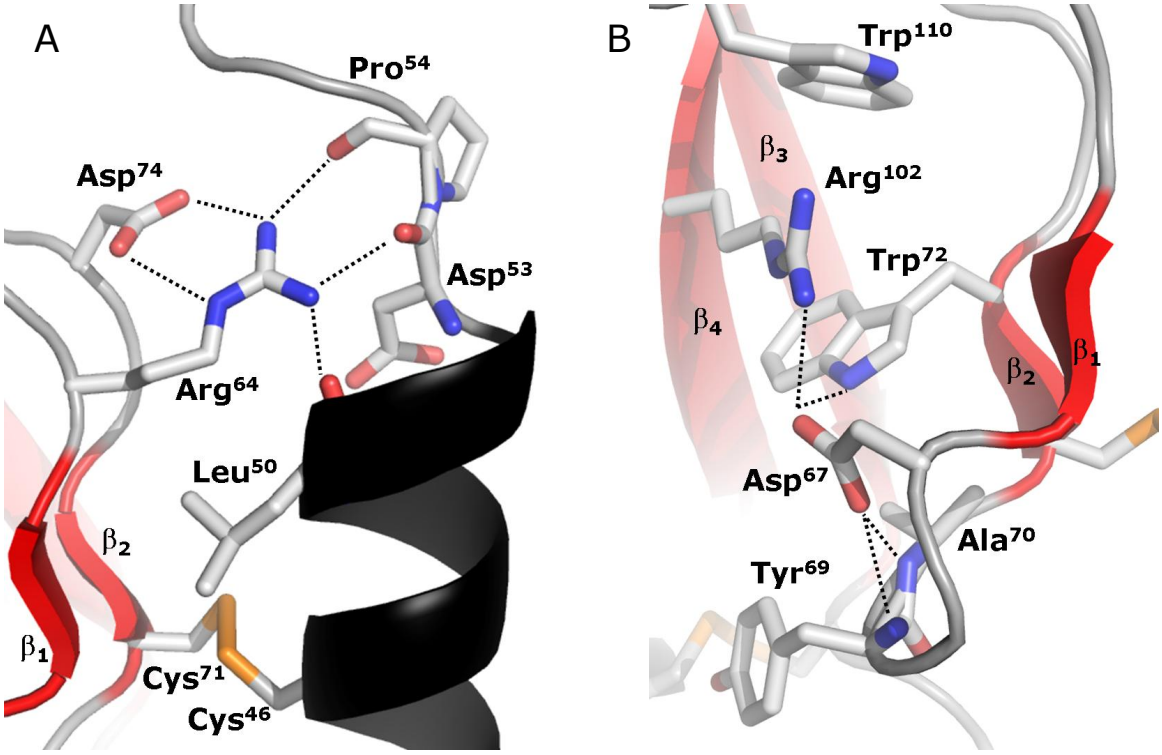

Figure 8. Intramolecular interactions of the GLP-1R ECD. (A) Stick representation of the disulphide bond between Cys^{46} on the α-helix (black) and Cys^{71} on β_2 (red) and the interactions of the guanidine group of Arg^{64} with Leu^{50}, Asp^{53}, Pro^{54} and Asp^{74}. **(B)** Stick representation of the residues Asp^{67}, Tyr^{69}, Ala^{70}, Trp^{72}, Arg^{102} and Trp^{110} (147). PDB ID: 3IOL.

Structural differences between the ECDs of GLP-1R, GIP-R and GCGR

Crystal structures of the ECDs of GLP-1R, GIP-R and GCGR are now available. The ECDs of GLP-1R and GIP-R have been crystallised in complex with their endogenous peptide ligands (161;175), but efforts to crystallise the glucagon-bound ECD of GCGR have so far been unsuccessful. However, GCGR ECD was recently crystallised in complex with the Fab fragment of a blocking antibody, mAb1 (165). The crystal structure revealed that mAb1 inhibits glucagon binding to the ECD by occupying a surface that covers the binding pocket.

The ECD of GLP-1R shares 34% and 46% sequence identity with the ECDs of GIP-R and GCGR, respectively, and the tertiary structure of the three ECDs is very similar (Figure 9A). However, small structural differences are observed in the N-terminal α-helix and in loop region L_6, which may contribute to ligand specificity. The most noticeable difference between the three structures is observed for the conformation of loop L_6. GLP-1R and GCGR contain an additional loop (L_6), which is not observed in the ECD of GIP-R. In addition, the conformation of L_6 is different in GLP-1R compared to GCGR, which Koth *et al.* explained by an additional amino acid (Leu^{118}) in GLP-1R compared to GCGR. However, as illustrated in Figure 9B, direct interactions between residues in L_6 of GCGR ECD and residues in the Fab fragment of mAb1 may better explain the altered conformation of loop L_6. In addition, Koth *et al.* explained the structural difference between the N-terminal α-helices of GCGR and GLP-1R by the presence of an additional residue, Phe^{33}, in the α-helix of GCGR ECD. However, as illustrated in Figure 9B, packing interactions between a symmetry-related Fab fragment and residues in the N-terminal α-helix of GCGR may also explain the slightly different conformation of this part of the ECD.

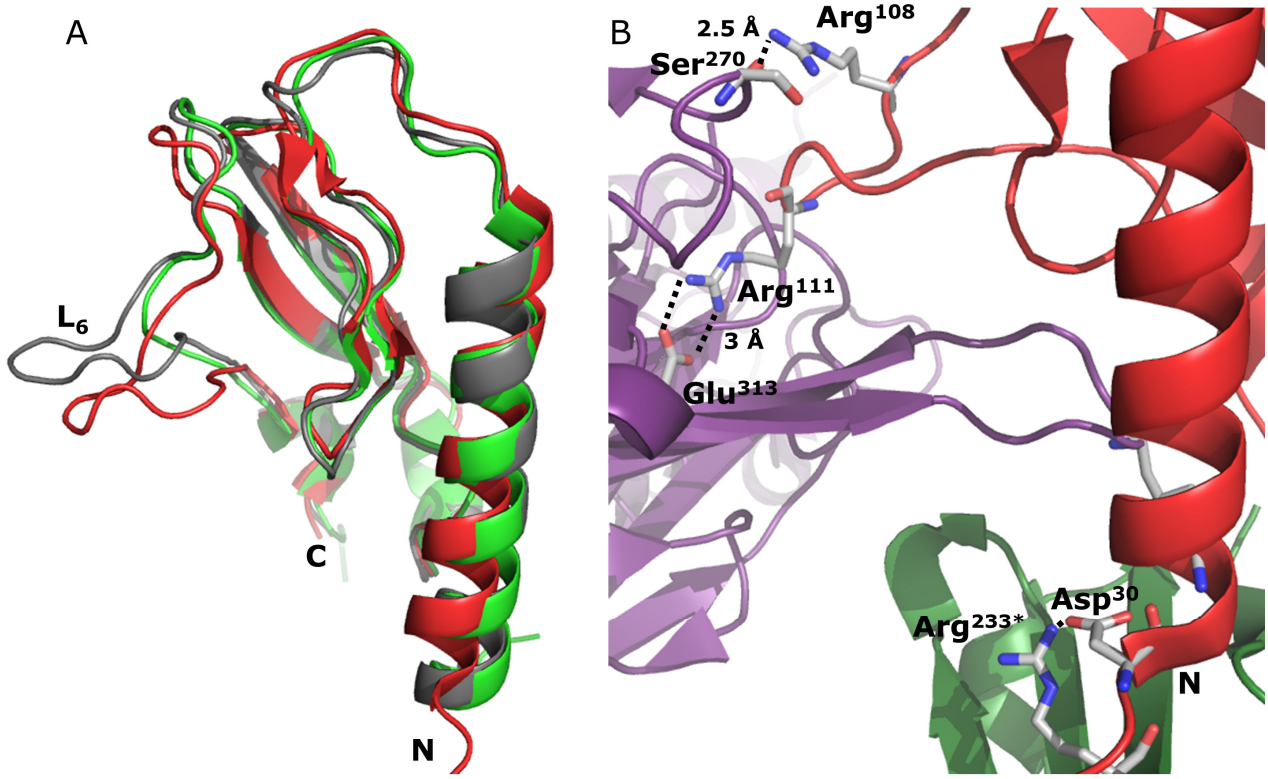

Figure 9. Structural differences between the ECDs of GLP-1R, GIP-R and GCGR. (A) The tertiary structure of GLP-1R ECD (grey, PDB ID: 3IOL) is highly similar to that of GIP-R (green, PDB ID: 2QKH) and GCGR (red, PDB ID: 4ERS). However, structural differences are observed in the N-terminal α-helix and in loop region L_6. **(B)** Some of the interactions between residues in the ECD of GCGR (red) and residues in the Fab fragment of mAb1 (purple). The guanidine groups of Arg^{108} and Arg^{111} in the ECD of GCGR interact with the backbone carbonyl of Ser^{270} and the carboxyl group of Glu^{313}, respectively, in the Fab fragment of mAb1. A hydrogen bond between Asp^{30} in the ECD of GCGR and Arg^{233*} in a symmetry-related Fab fragment (dark green) is also shown.

The transmembrane and C-terminal domain

Chimeric receptor studies and site-directed mutagenesis have been employed to characterise determinants of ligand binding and activation in the TM domain, loops and C-terminal tail of GLP-1R. A pair of polar residues in TM2 is a conserved feature among family B GPCRs, and they appear to be important for agonist binding and activation of several family B receptors (152;176;177). Neighbouring Arg and Ser residues make up a polar face of TM2 of GLP-1R (Figure 10), which may provide a surface for interaction with either GLP-1 or another TM helix during receptor activation. The latter is supported by site-directed mutagenesis studies on the PTH receptor, which suggest that Arg^{190} in TM2 could be functionally linked to Gln^{394} in TM7 (Figure 10) (178). In addition, an Arg^{190}-Ala mutation in GLP-1R reduced agonist- as well as antagonist affinity, suggesting that Arg^{190} is important for the general structure of the receptor rather than GLP-1 binding (179). It appears that polar residues in the TM domain are important for GLP-1 binding, as polar residues (Lys^{197}, Asp^{198}, Lys^{202}, Asp^{215} and Arg^{227}, Figure 10) in ECL1 and one polar residue in TM4 (Lys^{288}) probably contribute to the binding determinants of GLP-1R (180;181). However, nonpolar residues may also be involved in binding of GLP-1 to ECL1, as substitution of Met^{204} and Tyr^{205} with Ala (Figure 10) markedly reduced the affinity of GLP-1 (182). A disulphide bond connecting ECL1 and ECL2 seems to be a conserved feature among family A and family B GPCRs (125;183-185). Mutational studies on the VIP receptor suggest that this linkage is essential for high affinity ligand binding, possibly by stabilising an active receptor conformation (185). Similar observations were made for PTH-

1R, where mutation of Cys^{281} and/or Cys^{351} to serine reduced ligand binding and receptor expression levels (186). Mutational studies of GLP-1R suggest that a disulphide bond between ECL1 and ECL2 is not essential for GLP-1 binding, but ECL2 is probably positioned close to the binding pocket of the receptor (187). The importance of ECL2/TM5 in GLP-1 binding is further supported by two independent Ala-scans of this region (187;188). Taken together, residues in the loops and in the extracellular end of the TM-helices appear to be important for GLP-1 binding, but to less extent for binding of exendin-4 (182;189). Subtle differences in the binding modes to the TM domain may explain why the two ligands bind to the full-length GLP-1R with similar affinity despite the differential affinity for the isolated ECD. The existence of differences in the binding modes of GLP-1 and exendin-4 to the GLP-1R TM domain is also supported by the observation that N-terminal truncation of exendin-4 by two amino acids generates a high affinity antagonist (63). This is not observed for N-terminal truncation of GLP-1 (63).

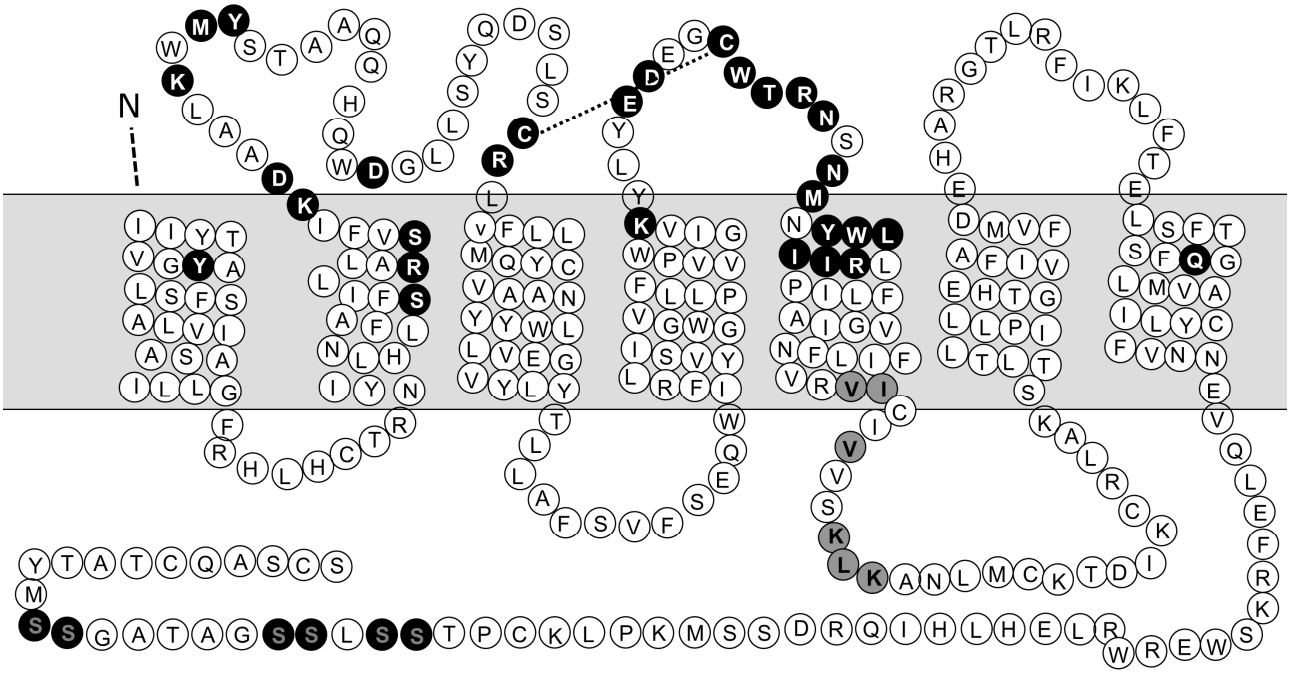

Figure 10. Snake diagram of the TM domain, loops and C-terminal tail of GLP-1R. Residues in the TM domain, which are experimentally determined to be important for peptide ligand binding and/or activation of the receptor are shown as white letters in black circles. Residues thought to be important for G_s-protein coupling are shown as black letters in grey circles and serine doubles involved in desensitisation and internalisation of the receptor are shown as grey letters in black circles. The putative disulphide bond that connects ECL1 and ECL2 is indicated with a dashed line.

Long before GLP-1R was cloned, it was recognised that GLP-1 increases the production of cAMP in islet cell lines (79;168). Shortly after the rat GLP-1R was cloned, it was shown that activation of the receptor leads to activation of AC and PLC, which increases cellular concentrations of cAMP and Ca^{2+}, respectively (190). It is now well established that GLP-1R can signal through multiple G-protein signalling pathways-at least in some cell lines. GLP-1R can couple to $G_{s\alpha}$, $G_{q\alpha}$ and $G_{i\alpha}$ in Chinese hamster ovary (CHO) cells (191), depending on the ligand (functional selectivity). In addition, GLP-1R can signal independently of G-proteins, that is, the receptor can activate the mitogen-activated protein (MAP) kinase pathway, leading to phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) through the recruitment of β-arrestins (192;193). The receptor preferably couples to $G_{s\alpha}$ (194), and the

subsequent rise in cAMP levels has a direct effect on insulin secretion (79). Oxyntomodulin is a 37 amino acid peptide that contains the sequence of glucagon followed by a C-terminal extension of 8 amino acids (Figure 1). Oxyntomodulin acts as a full agonist of GLP-1R in mediating a cAMP response (195), but as a partial agonist in mediating a Ca^{2+} response in CHO cells (194) and in recruitment of β-arrestins to GLP-1R in human embryonic kidney (HEK) cells (195). Specific determinants for the coupling of GLP-1R to G_s are thought to be located mainly in the cytoplasmic end of TM5 and in the N-terminal part of ICL3 (Figure 10). Mutation of hydrophobic residues in the cytoplasmic end of TM5 (Val^{327}, Ile^{328}) and in ICL3 (Val^{331}) significantly lowered the production of cAMP without reducing receptor expression (196). In addition, deletion studies of GLP-1R suggest that a conserved Lys-Leu-Lys motif in the N-terminal part of ICL3 plays a critical role for G_s-protein coupling (197), which is further supported by studies of synthetic peptide sequences derived from ICL3 of GLP-1R (198). Conversely, the C-terminal part of ICL3 may be involved in coupling to the $G_{i\alpha}$- subunit (198).
The truncation experiments described in Study III indicate that the distal part of the GLP-1R C-terminal tail is not involved in the signal transduction process. Similar observations have been made for other family B GPCRs (199;200). Instead, phosphorylation of three serine doublets in the distal part of the GLP-1R C-terminal (Ser^{441}/Ser^{442}, Ser^{444}/Ser^{445} and Ser^{451}/Ser^{452}, Figure 10) appears to be important for desensitisation and internalisation of the receptor (201).

Small molecule ligands

GLP-1 analogues have established a new class of drugs for the treatment of T2D, but have the disadvantage of not being orally available. Thus, small molecule agonists that target GLP-1R are preferable, but difficult to identify and develop, probably due to the large and open binding pocket of family B GPCRs. However, in recent years, several small molecule ligands have been discovered for GLP-1R. Most of these are merely used as research tools, but some may represent pharmacophores that can be optimised further for clinical development (202).

Small molecule antagonists

T-0632 was originally identified as an antagonist for the cholecystokinin (CCK) receptor (203), but was later shown to act as a non-competitive antagonist for GLP-1R (204). T-0632 binds to the human GLP-1R with an IC_{50}-value of 1.2 μM, and was the first reported small molecule antagonist of GLP-1R. The authors identified a single residue, Trp^{33}, in the ECD of GLP-1R that was involved in T-0632 binding, but not in GLP-1 binding (204). Trp^{33} does not make direct contact with GLP-1 when bound to ECD. The residue is positioned approximately 8 Å from the peptide binding pocket (162;175), which could explain the non-competitive, yet antagonistic properties of T-0632. A similar observation was made for the competitive antagonist olcegepant, which binds to the calcitonin gene-related peptide (CGRP) receptor (CGRP-R) ECD and blocks access to the peptide binding pocket (205). Other small molecule antagonists have been shown to target the TM domain of family B receptors. Site-directed mutagenesis of several residues in the TM domain of the rat GCGR identified residues in TM2 and TM3, whose substitution with Ala decreased the binding affinity of a small molecule GCGR antagonist, while preserving glucagon binding (206). Moreover, residues in the extracellular end of TM3 and TM5 were shown to determine receptor-subtype selectivity of a small molecule antagonist of the CRFR1 receptor (207).
A few small molecule antagonists have been identified that target GCGR as well as GLP-1R, suggesting that the two receptors may share a similar non-peptide binding pocket (208-210). In addition, the naturally occurring flavonoid, catechin, was recently found to act as a non-competitive antagonist of GLP-1R (211).

Small molecule agonists

A number of small molecules have been identified that activate GLP-1R (194;211-214) (Figure 11). A screening of ~250.000 compounds identified a small molecule that stimulated cAMP production in transfected BHK cells expressing GLP-1R (213). Subsequent optimisation generated a full agonist, Compound 2 (Figure 11A) that stimulated cAMP production with an EC_{50} value of 0.10 μM (213). Compound 2 was also found to potentiate insulin secretion from isolated mouse pancreatic islets in a glucose dependent manner, whereas neither GLP-1 nor Compound 2 potentiated insulin secretion from islets of GLP-1R knockout mice (213). Other small molecule agonists like Boc5 (Figure 11B) and BETP (Figure 11C), have been identified that mimic the effects of GLP-1 *in vitro* and *in vivo* (212;214). The EC_{50} values of Boc5 and BETP were found to be 2.73 μM and 0.66 μM, respectively (212;214). A three-dimensional model structure of GLP-1R was used to predict the putative binding site of Boc5 to be located near the extracellular end of TM3 and TM4 (215). However, it is worth noting that the agonistic effect of Boc5, but not the agonistic effect of Compound 2 or BETP, was blocked by the GLP-1R antagonist exendin-4(9-39) (212-214). Hence, it appears that Boc5 binds to- or near the orthosteric binding site, whereas Compound 2 and BETP may bind to an allosteric site in GLP-1R. A series of allosteric modulators of GLP-1R was reported recently based on naturally occurring as well as synthetic flavonoids (194;211). It has previously been reported that quercetin (Figure 11D), a naturally occurring flavonoid, has antidiabetic effects in rodents (216). Sexton *et al.* extended this work and showed that quercetin and quercetin-like compounds can modulate GLP-1R action in a ligand– and pathway dependent manner (194;211).

Unfortunately, most the small molecule GLP-1R agonists developed to date are unsuitable for oral administration. Compound 2 and BETP are chemically unstable in the presence of nucleophiles, and the physiochemical properties of Boc5 render this compound incompatible with oral administration (202). However, Transtech Pharma has developed a series of potent small molecule GLP-1R agonists of which the leading compound, TTP054, is currently under clinical evaluation in phase II trials (202).

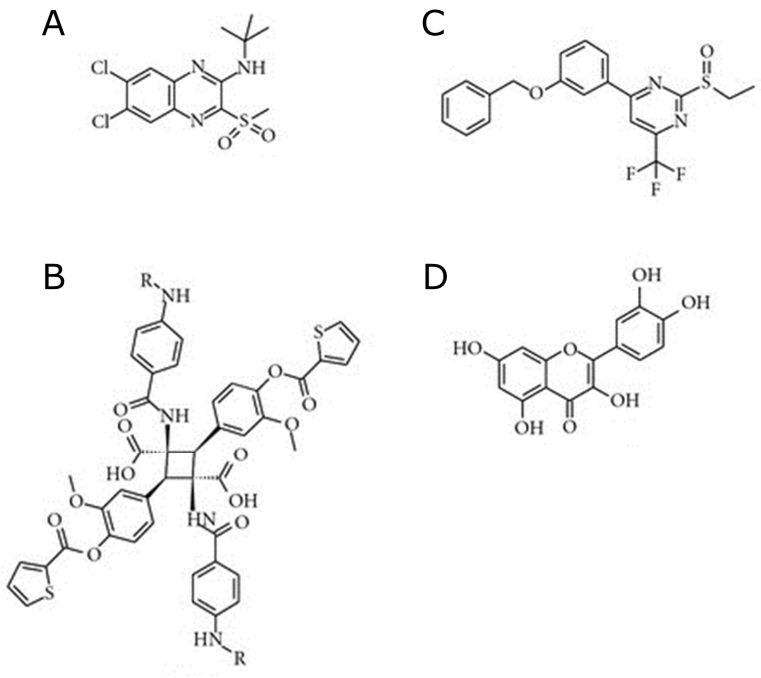

Figure 11. Structures of small molecule ligands that target GLP-1R. Chemical structures of small molecule GLP-1R ligands, based on four different scaffolds. **(A)** Compound 2 (213), **(B)** Boc5 (212), **(C)** BETP (214) and **(D)** Quercetin (194).

Allosteric properties of small molecule ligands

According to the classical definition, a positive allosteric modulator is a compound that binds to an allosteric site of a receptor, and enhances the effect of the endogenous agonist, without having any agonist activity itself (217). However, several compounds that act as agonists as well as allosteric modulators have now been identified (213;214;218;219) and termed ago-allosteric modulators (220). Compound 2 and BETP are good examples of ago-allosteric modulators of GLP-1R. Both compounds stimulate cAMP production in cells expressing GLP-1R and both compounds increase glucose-dependent insulin secretion from isolated islets (213;214). The allosteric nature of the two small molecule agonists is supported by the observation that Compound 2 significantly increases the affinity of GLP-1 for GLP-1R (213), whereas BETP significantly increases the efficacy of GLP-1 (214) compared to GLP-1 alone. In addition, BETP and Compound 2 appear to increase oxyntomodulin affinity for GLP-1R and oxyntomodulin-mediated activation of GLP-1R in a pathway-specific manner (194;221). Hence, BETP and Compound 2 increase oxyntomodulin-mediated signalling through the cAMP pathway, but not through other signalling pathways (194;221). Emerging evidence indicate that functional selectivity may be a relatively common feature of allosteric modulators of GLP-1R. The naturally occurring, small molecule GLP-1R ligands quercetin and catechin were recently demonstrated to display functional selectivity. Quercetin potentiates the release of Ca^{2+} in a peptide-agonist dependent manner. That is, quercetin is unable to activate GLP-1R in the absence of a peptide agonist, but potentiates the release of Ca^{2+} induced by high affinity agonists like GLP-1 and exendin-4, but not by oxyntomodulin. Quercetin has no effect on peptide-induced signalling through other pathways (194). Conversely, catechin decreases GLP-1 efficacy in the cAMP signalling pathway, but has no significant effect on other peptide agonists or other pathways (211).

Ago-allosteric modulators represent a new subclass of allosteric ligands, but more studies are required to fully understand the pharmacological potential of these dual-acting small molecules. The ago-allosteric nature and complex signalling profile of these ligands does however serve as an excellent tool to study GPCR pharmacology. It is now widely accepted that GPCR ligands can stabilise distinct receptor conformations and in this way elicit different signal transduction pathways (222). Hence, Compound 2 and BETP may stabilise a receptor conformation that favours binding of oxyntomodulin, which in turn favours signalling through the cAMP pathway. It has previously been suggested that ago-allosteric modulators could act in dimeric receptor setting (220), but this was recently disproved for GLP-1R (223). It was shown that GLP-1R forms homodimers via a TM4/TM4 interface similar to other family B GPCRs (224;225). Disruption of the dimer reduced peptide- and small molecule agonist potency in various signalling pathways, but the allosteric effects of Compound 2 and BETP were maintained, indicating that allosteric modulation of GLP-1R occurs in a single receptor setting (223).

RESULTS AND DISCUSSION

The scientific work included in this thesis is dedicated to studying interactions between GLP-1R and receptor agonists. This section contains four studies (Study I-IV). The work included in Study I and II was published in 2010 and 2011, respectively, and Study III will be submitted to 'Peptides' in the near future. The two papers and the manuscript are included in the thesis immediately after a short introduction to each of the studies. Study IV contains unpublished data, but some of the results are included in a co-authored paper that is currently in preparation (226).

Study I – Crystal structure of GLP-1-bound ECD of GLP-1R

A part of the thesis work focused on identifying specific interactions between GLP-1 and the ECD of GLP-1R, as well as investigating the differential affinity of GLP-1 and exendin-4 for the ECD. The work resulted in a crystal structure of GLP-1 in complex with the ECD of GLP-1R (Figure 12A), which was published in Journal of Biological Chemistry in 2010. Throughout this study, GLP-1 and exendin-4 residues are designated with * and **, respectively.

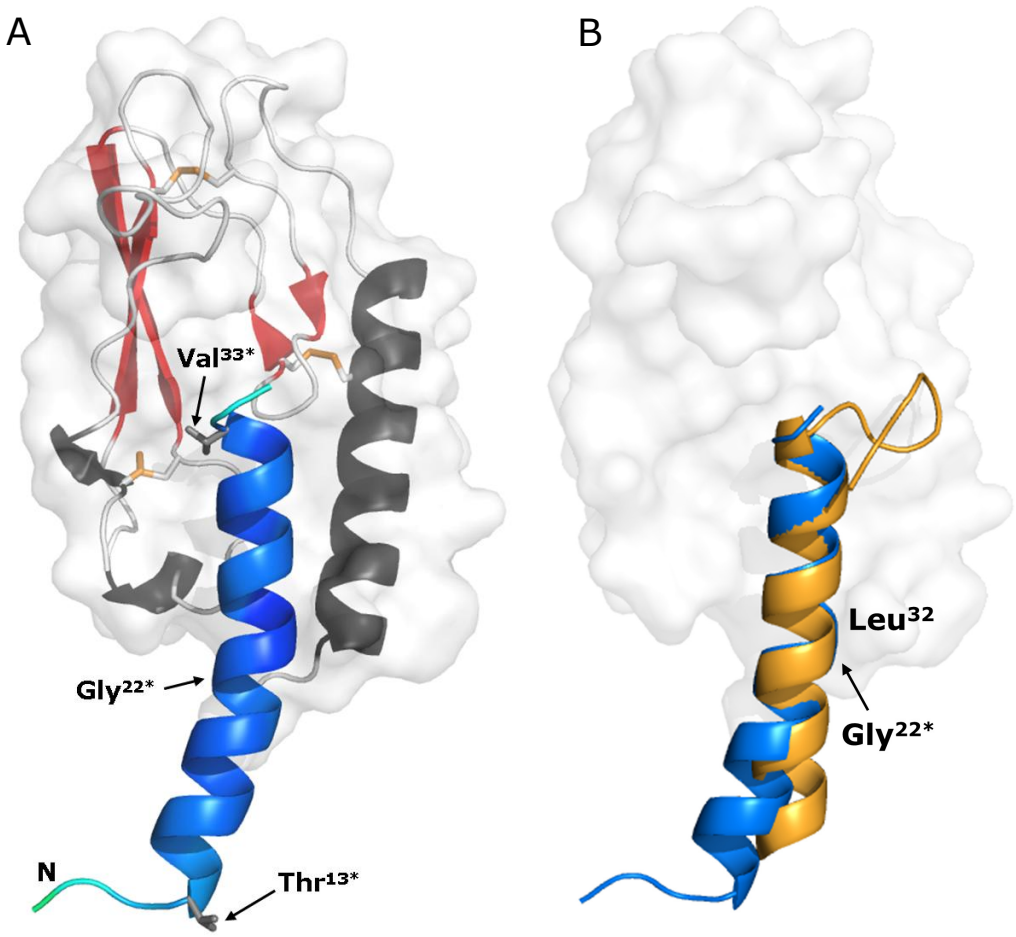

Figure 12. Structure of GLP-1-bound ECD of GLP-1R. (A) GLP-1 (blue) bound to the ECD of GLP-1R (α-helix in black, β-strands in red, loops in grey and surface in white). Disulphide bonds are shown as orange sticks. GLP-1 is a continuous α-helix from Thr^{13*} to Val^{33*} with a kink around Gly^{22*}. Thr^{13*} and Val^{33*} are shown as sticks. **(B)** GLP-1 (blue, PDB IB: 3IOL) versus exendin-4(9-39) (orange, PDB ID: 3C59)-bound structure of the ECD (surface in white). Leu^{32} of the ECD and Gly^{22*} of GLP-1 are indicated.

In 2008, Runge *et al.* solved the crystal structure of GLP-1R ECD in complex with exendin-4(9-39) (162), which for the first time elucidated ligand binding to GLP-1R on a detailed molecular level. The crystal structure solved during the Ph.D. work showed that GLP-1 is a continuous α-helix from Thr^{13*} to Val^{33*} when bound to the ECD, but the helix has a distortion of the backbone around Gly^{22*} (Figure 12). This distortion is not observed in the exendin-4(9-39)-bound structure (Figure 12B). However, it is unclear whether a kinked helix is important for GLP-1 binding to GLP-1R or whether the kinked helix is a result of crystal packing. Site-directed mutagenesis of the full-length GLP-1R subsequently demonstrated a ligand-specific effect of the Leu^{32}-Ala mutation. Leu^{32} in the ECD of GLP-1R is positioned right next to the helix kink of GLP-1 (Figure 12B). This may be coincidental, but structural difference between GLP-1 and exendin-4(9-39) (kinked versus straight helix) could also explain the differential effect of the Leu^{32}-Ala mutation on ligand binding.
Study I was performed with help from Steffen Reedtz-Runge, who undertook the data collection and the structural determination of the complex. I did all the experimental work and wrote the manuscript.

GLP-1 versus exendin-4

Exendin-4 is a 39 amino acid peptide originally isolated from the venom of the lizard *Heloderma suspectum*, also known as the Gila monster (65). GLP-1 and exendin-4 are approximately 50% identical (Figure 13C).

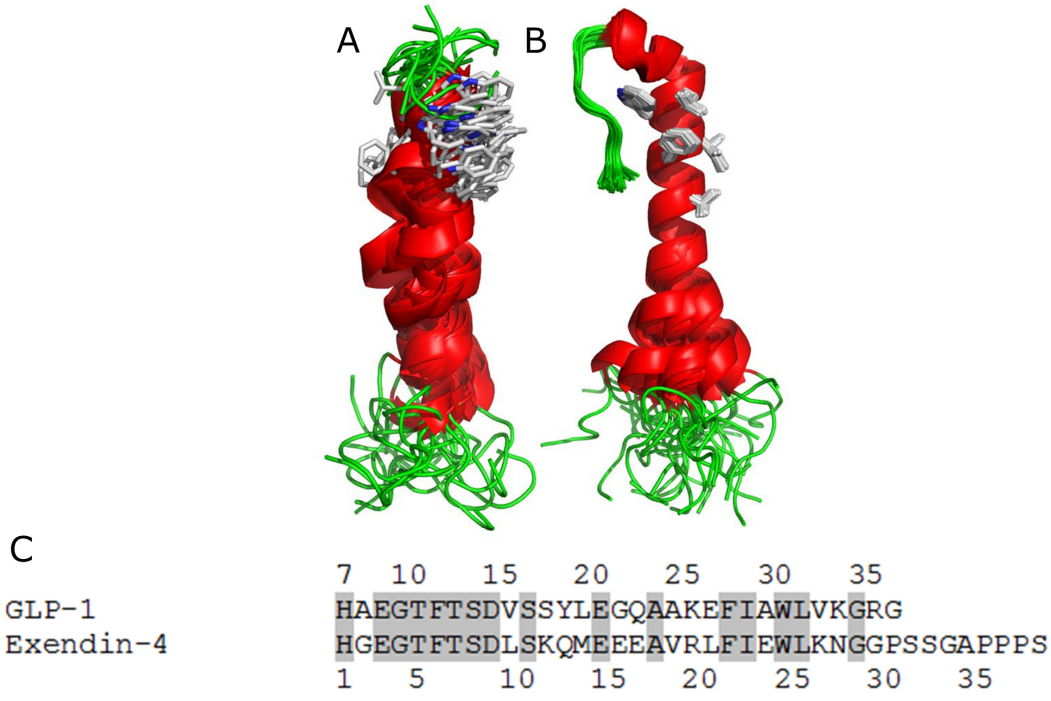

Figure 13. GLP-1 versus exendin-4. (A) Solution NMR structure ensemble of GLP-1 in 35% trifluoroethanol (TFE) (PDB ID: 1D0R, (227)). **(B)** Solution NMR structure ensemble of exendin-4 in 30% TFE (PDB ID: 1JRJ, (228)). The α-helices and loops are shown in red and green, respectively. Residues shown as sticks: Phe^{28*}, Ile^{29*}, Trp^{31*} and Leu^{32*} of GLP-1 and Val^{19**}, Phe^{22**}, Ile^{23**}, Trp^{25**} and Leu^{26**} of exendin-4. Leu^{21**}-Pro^{38**} of exendin-4 form a tertiary structure known as the Trp-cage, which shields the side chain of Trp^{25**} from solvent exposure (228). **(C)** Sequence alignment of GLP-1 and exendin-4, with conserved residues highlighted in grey. The highest degree of identity is found in the N-terminal part of the peptides, where 9 out of 11 residues are conserved. Residue number 1 of exendin-4 corresponds to residue number 7 of GLP-1.

As mentioned, GLP-1, exendin-4 and the N-terminally truncated exendin-4(9-39) bind to the full-length GLP-1R with almost similar affinity (IC_{50} ~0.5 nM). However, GLP-1 binds to the isolated ECD with reduced affinity (IC_{50} > 500 nM), whereas exendin-4 and exendin-4(9-39) maintain high affinity for the isolated ECD (IC_{50} = 6 nM) (173;174). This discrepancy in binding modes was initially explained by an additional interaction between the Trp-cage motif of exendin-4 and the ECD of GLP-1R (229). In TFE, the C-terminal extension of exendin-4 folds back onto the central part of the peptide and forms a protein-like fold known as the Trp-cage (Figure 13B) (228), which is not observed in GLP-1 (Figure 13A). In fact, the helical portion of GLP-1 is less stable than that of exendin-4 in TFE (228), and GLP-1 is almost devoid of secondary structure in aqueous buffer (230). It was later shown that the differential affinity for the GLP-1R ECD can be explained almost entirely by divergent residues in the central helical parts of GLP-1 and exendin-4 (174). These divergent residues (Glu^{21*}-Arg^{36*} in GLP-1 and Glu^{15**}-Gly^{30**} in exendin-4) render exendin-4 more helical in solution compared to GLP-1 (174;228), which in turn increases its affinity for the ECD of GLP-1R.

PAPER I

CRYSTAL STRUCTURE OF GLP-1 IN COMPLEX WITH THE EXTRACELLULAR DOMAIN OF THE GLP-1 RECEPTOR

⌘ *Author's Choice*

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Crystal Structure of Glucagon-like Peptide-1 in Complex with the Extracellular Domain of the Glucagon-like Peptide-1 Receptor*[S]

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GLP-1 (glucagon-like peptide-1) is an incretin released from intestinal L-cells in response to food intake. Activation of the GLP-1 receptor potentiates the synthesis and release of insulin from pancreatic β-cells in a glucose-dependent manner. The GLP-1 receptor belongs to class B of the G-protein-coupled receptors, a subfamily characterized by a large N-terminal extracellular ligand binding domain. Exendin-4 and GLP-1 are 50% identical, and exendin-4 is a full agonist with similar affinity and potency for the GLP-1 receptor. We recently solved the crystal structure of the GLP-1 receptor extracellular domain in complex with the competitive antagonist exendin-4(9–39). Interestingly, the isolated extracellular domain binds exendin-4 with much higher affinity than the endogenous agonist GLP-1. Here, we have solved the crystal structure of the extracellular domain in complex with GLP-1 to 2.1 Å resolution. The structure shows that important hydrophobic ligand-receptor interactions are conserved in agonist- and antagonist-bound forms of the extracellular domain, but certain residues in the ligand-binding site adopt a GLP-1-specific conformation. GLP-1 is a kinked but continuous α-helix from Thr[13] to Val[33] when bound to the extracellular domain. We supplemented the crystal structure with site-directed mutagenesis to link the structural information of the isolated extracellular domain with the binding properties of the full-length receptor. The data support the existence of differences in the binding modes of GLP-1 and exendin-4 on the full-length GLP-1 receptor.

GLP-1 (glucagon-like peptide-1) is a peptide hormone produced by post-translational processing of proglucagon in the intestinal L-cells (1). GLP-1 is an incretin that potentiates the synthesis and release of insulin from pancreatic β-cells in a glucose-dependent manner (2) and has a number of other beneficial effects that contribute to reducing blood sugar (3–6). In addition, GLP-1 has been shown to reduce body weight (3, 5, 7), which is favorable for many people with type 2 diabetes. Hence, GLP-1-based therapies are becoming increasingly attractive for the treatment of type 2 diabetes. The actions of GLP-1 are mediated through the GLP-1 receptor (GLP-1R),[3] which is a seven-transmembrane G protein-coupled receptor (GPCR) coupled to adenylyl cyclase (8). The human GLP-1R was first cloned in 1992 and belongs to class B of GPCRs (9). This class includes a wide range of receptors for peptide hormones like glucagon, GLP-2 (glucagon-like peptide-2), glucose-dependent insulinotropic polypeptide (GIP), pituitary adenylyl cyclase-activating polypeptide (PACAP), vasoactive intestinal polypeptide, secretin, calcitonin, corticotrophin-releasing factor, and parathyroid hormone (PTH) (10). The receptors are distinguished by their large extracellular N-terminal domain (ECD), which is important for ligand binding and selectivity (11, 12). The current binding model suggests a two-domain mechanism where the C-terminal part of the ligand binds the ECD and the N-terminal part of the ligand binds the extracellular loops and transmembrane α-helices (TM domain), which leads to receptor activation (reviewed in Ref. 13).

The first structure of an isolated ECD of a class B GPCR was solved recently by NMR spectroscopy (14). Subsequently, structures of ligand-bound ECDs were solved, including the ECD of the human type 1 corticotrophin-releasing factor receptor 1 (15), the human type 1 PACAP receptor (16), the human GIP receptor (17), the human GLP-1R (18), and the human type 1 PTH receptor (19). The ECDs of class B receptors have a common structure, the secretin recognition fold, which is stabilized by three conserved disulfide bonds and five conserved residues (Asp[67], Trp[72], Pro[86], Gly[108], and Trp[110] in GLP-1R). The receptor-bound ligands are primarily in α-helical con-

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⌘ *Author's Choice*—Final version full access.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

The atomic coordinates and structure factors (code 3IOL) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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[3] The abbreviations used are: GLP-1R, GLP-1 receptor; GPCR, G protein-coupled receptor; GIP, glucose-dependent insulinotropic polypeptide; PACAP, pituitary adenylyl cyclase-activating polypeptide; PTH, parathyroid hormone; ECD, extracellular N-terminal domain; TM domain, extracellular loops and transmembrane α-helices.

formation, and the C-terminal part of the ligands binds the ECD in agreement with the two-domain binding mechanism. Several receptor models have been proposed for full-length class B receptors (20–23). However, the orientation of the ECD relative to the TM domain is uncertain.

Exendin-4 is a 39-amino acid peptide, which was originally isolated from the venom of the lizard *Heloderma suspectum* (24). GLP-1 and exendin-4 are 50% identical, and exendin-4 is a full agonist with similar affinity and potency for the full-length GLP-1R (25). However, structure-activity studies have demonstrated interesting differences between the binding modes of GLP-1 and exendin-4. 1) The isolated ECD binds exendin-4 with high affinity (IC_{50} of 6 nM) and GLP-1 with low affinity (IC_{50} >500 nM) (26, 27). 2) GLP-1 binding is more sensitive to site-directed mutagenesis of the TM domain compared with exendin-4 binding (28–30). 3) GLP-1 is much more sensitive to N-terminal truncation than exendin-4 (31). N-terminally truncated exendin-4 variants maintain high affinity but are unable to activate GLP-1R, *i.e.* competitive antagonist, whereas N-terminal truncation of GLP-1 severely affects both binding and activation (31). Clearly, the interaction between the N-terminal part of GLP-1 and the TM domain is critical for binding and activation of GLP-1R.

Exendin-4(9–39) is a truncated form of exendin-4, and a competitive antagonist that maintains high affinity for GLP-1R through interactions with the ECD (IC_{50} value of 6 nM for the isolated ECD) (27, 32). We recently solved the crystal structure of the GLP-1R ECD in complex with exendin-4(9–39) (18). Exendin-4(9–39) is α-helical in the ECD-bound conformation except for the C-terminal segment, the so-called Trp cage (33, 34). In solution, the helical propensity of exendin-4 is higher than that of GLP-1 (27, 33). Biophysical studies showed a positive correlation between α-helical propensity in solution and affinity for the GLP-1R ECD (27). Moreover, charged residues of exendin-4 interact with the ECD in a manner not possible for GLP-1 (18). Hence, the high affinity of exendin-4 for the ECD may be a combination of high helical propensity in solution and unique receptor interactions.

Here, we report the crystal structure of the GLP-1R ECD in complex with its endogenous agonist GLP-1. We supplemented the crystal structure with site-directed mutagenesis to link the structural information of the isolated ECD with the binding properties of the full-length receptor.

EXPERIMENTAL PROCEDURES

Protein and Peptide Preparation—The GLP-1R ECD was prepared as described previously (27). Briefly, N-terminal His_6-tagged ECD was expressed in *Escherichia coli* inclusion bodies, isolated as inclusion body protein, solubilized in guanidine-HCl and dithiothreitol, dialyzed against guanidine-HCl to remove the dithiothreitol, and refolded using L-Arg and a 1:5 molar ratio of reduced and oxidized glutathione. The refolded ECD was purified by hydrophobic interaction chromatography and size exclusion chromatography in 10 mM Tris-HCl, pH 7.5, 0.1 M Na_2SO_4, 2% glycerol. The His_6 tag was removed by thrombin cleavage. The purified GLP-1R ECD consisted of four amino acids, Gly-Ser-His-Met, of the linker attached to the N-terminus of ECD (Arg^{24}–Tyr^{145}), M_r = 14,723 g/mol after removal of the His_6 tag. Native GLP-1(7–37)-OH was synthesized as described previously (12).

Purification and Crystallization of the GLP-1-bound Extracellular Domain—The purified GLP-1R ECD was concentrated to 1.2 mg/ml, mixed with 3-fold molar excess of GLP-1(7–37) (dissolved in 50 mM Tris-HCl, pH 7.5), and incubated overnight at 4 °C. The GLP-1-bound ECD was purified by size exclusion chromatography on a Superdex 75 column in 10 mM Tris-HCl, pH 7.5, at a flow rate of 0.3 ml/min and characterized by SDS-PAGE (supplemental Fig. S1). The complex was concentrated to 4 mg/ml and crystallized by hanging drop vapor diffusion. The crystallization conditions were initially identified using the Crystal Screen from Hampton Research and subsequently optimized to 0.1 M *N*(2-acetamido)iminodiacetic acid, pH 6.9, 14 volume % (±)-2-methyl-2,4-pentanediol and 9 mM *n*-decyl-β-D-thiomaltoside. Single crystals were flashed cooled in liquid N_2 using 30% glycerol in the cryo solution.

Data Collection and Structure Determination—Diffraction data were collected from a single crystal using beamline 1911-3 at MAX-lab (Lund, Sweden). The data were integrated and scaled using XDS (35). The crystals belonged to space group $P2_122_1$ with the unit cell dimensions a = 35.7 Å, b = 42.7 Å, and c = 95.1 Å. The phases and electron density map were obtained by molecular replacement using Phaser running in the CCP4 program interface with one complex in the asymmetric unit. Refinement was done using COOT (36) and REFMAC5 (37). Well defined electron density was obtained for GLP-1 residues Thr^{11*}–Val^{33*} and for ECD residues Val^{30}–Glu^{128}. Poor density was observed for GLP-1 residues Gly^{10*}, Lys^{34*}, and Gly^{35*} and ECD residues Thr^{29} and Asn^{115}. No electron density was observed for His^{7*}–Glu^{9*} and Arg^{36*}–Gly^{37*} of GLP-1. Our final structure contains GLP-1 residues Gly^{10*}–Gly^{35*} and ECD residues Thr^{29}–Glu^{128}, but it should be noted that the conformation of Lys^{34*} of GLP-1 is very uncertain, and because of the poor density we have chosen to show Asn^{115} as Gly. The final GLP-1-bound ECD structure has 116 residues in preferred regions, 5 in allowed regions and only 1 outlier (Glu^{68} in the β-turn between β_1 and β_2 shown in Fig. 1*A*) in the Ramachandran plot. The structure containing 73 water molecules and a detergent molecule (*n*-decyl-β-D-thiomaltoside) has a working *R*-factor of 0.181% and a free *R*-factor of 0.226%. Data collection and refinement statistics are summarized in Table 1. Coordinates and structure factors are deposited in the Protein Data Bank under accession code 3IOL. Molecular graphics were prepared in PyMOL (46).

Receptor Constructs—The cDNA encoding the human GLP-1R was originally obtained from Dr. B. Thorens (9) and subcloned into the mammalian expression vector pcDNA3.1/v5-His-TOPO® (Invitrogen). The presence of the C-terminal His_6 tag was previously shown not to influence the functional response of the receptor (12). Site-directed mutagenesis of GLP-1R was done using QuikChange™ (Stratagene). Plasmid DNA was generated using the NucleoBond® Xtra Maxi Plus kit (Macherey-Nagel), and the desired mutations were confirmed by dideoxynucleotide sequencing.

Cell Culture and Receptor Expression—Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 10 volume

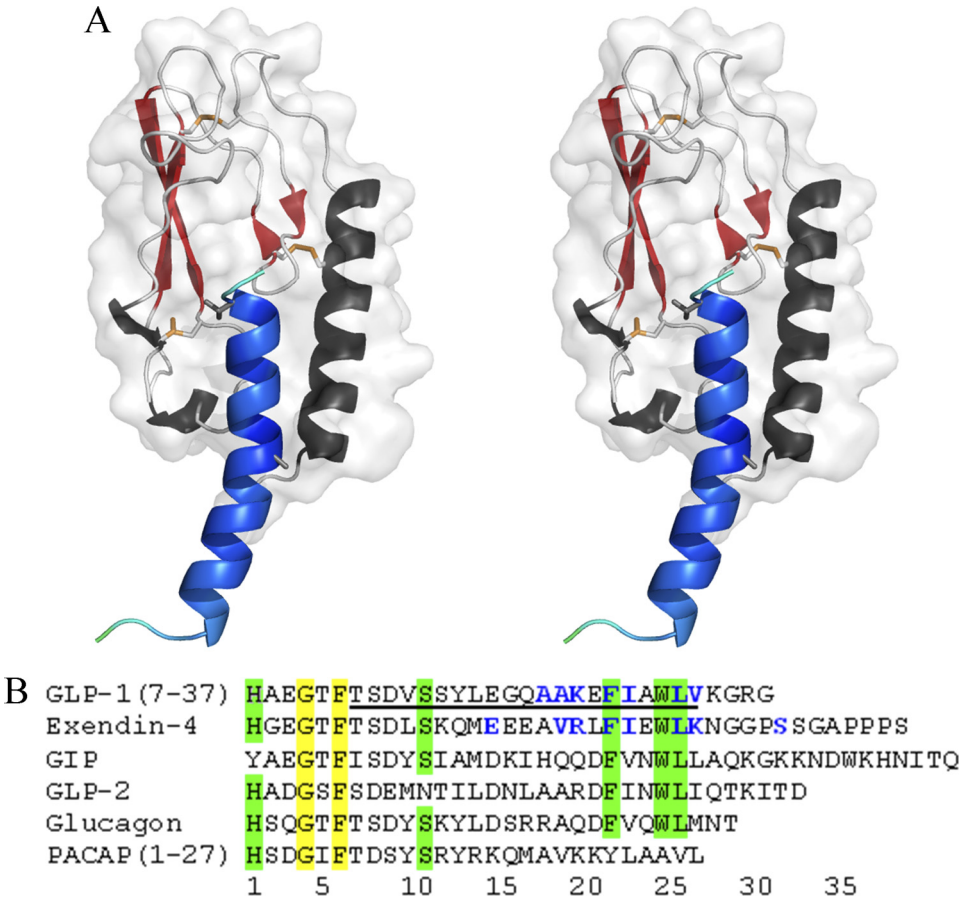

FIGURE 1. **Structure of the GLP-1-bound ECD of the GLP-1R.** *A*, stereoview of GLP-1 (*blue*) bound to the ECD of the GLP-1R (α-helix in *black*, β-strands in *red*, and loops in *gray*). Disulfide bridges are shown as *orange sticks*. Residues Cys^{62}–Asp^{67} (β_1) and Ala^{70}–Gly^{75} (β_2) constitute the first region of antiparallel β-sheets, and the second region is comprised of residues Gly^{78}–Ser^{84} (β_3) and His^{99}–Thr^{105} (β_4), which is shown in *red*. Our final structure contains GLP-1 residues Gly^{10}*–Gly^{35}*. The residues that interact with GLP-1R ECD lie within Ala^{24}* and Val^{33}*, which are shown as *sticks*. *B*, sequence alignment of GLP-1, exendin-4, GIP, GLP-2, glucagon, and PACAP(1–27). Fully conserved residues are highlighted in *yellow*, and partially conserved residues are highlighted in *green*. The residues of GLP-1 and exendin-4 that interact with GLP-1R ECD are colored *blue*. The *underlined residues* symbolize residues of GLP-1 in α-helical conformation when bound to the ECD. Residue number 1 of exendin-4 corresponds to residue number 7 of GLP-1.

% fetal bovine serum and 1 volume % penicillin/streptomycin (100 units/ml) in T175 flasks. HEK293 cells were transiently transfected with 21 μg of GLP-1R DNA using the FuGENE™ transfection reagent (Roche Applied Science), harvested 24 h after transfection, and used directly in functional experiments or plasma membrane preparations as described previously (12).

Functional Assay—Transiently transfected HEK293 cells expressing wild-type GLP-1R or mutant receptors were harvested and resuspended in assay buffer (Flashplate®, PerkinElmer Life Sciences) to a cell density of 2.4×10^6 cells/ml. GLP-1(7–37)-acid and exendin-4 were diluted in phosphate-buffered saline with 0.02 volume % Tween 20. Cells in assay buffer (50 μl) and GLP-1 or exendin-4 (50 μl) were mixed in 96-well FlashPlates® (PerkinElmer Life Sciences), gently agitated for 5 min, and incubated for 25 min at room temperature. The resulting intracellular level of cAMP was measured according to supplier's manual and analyzed by nonlinear regression/sigmoidal dose-response fitting using Prism 5.0® (GraphPad Software, Inc.).

Receptor Binding Assay—Freshly thawed plasma membrane preparations from transiently transfected HEK293 cells expressing GLP-1R (20 μg protein/well) were pulled through a 25-gauge needle three times and diluted in assay buffer (50 mM HEPES, 5 mM $MgCl_2$, 5 mM EGTA, 0.005 volume % Tween 20, pH 7.4). GLP-1 and exendin-4 were diluted in assay buffer. The concentration range was 1 pM to 100 nM for GLP-1 and exendin-4. ^{125}I-GLP-1(7–36)-amide (2.2 Ci/μmol) was dissolved in assay buffer and added at 50,000 cpm per well to a final concentration of 50 pM. Nonspecific binding was determined with 1 μM GLP-1. Membrane preparation and radioligand were mixed in 96-well 0.65-μm filter plates (Millipore) with either diluted GLP-1 or exendin-4 and incubated for 1 h at 37 °C. Subsequently, bound and unbound radioligands were separated by vacuum filtration (Millipore vacuum manifold). The filters were washed twice in 100 μl of cold assay buffer and left to dry. Data were analyzed by nonlinear regression, and the expression level (B_{max}) was calculated using Prism 5.0® (GraphPad Software, Inc.).

RESULTS AND DISCUSSION

Purification, Crystallization, and Structure Determination—The GLP-1R ECD was expressed in *E. coli* inclusion bodies, refolded, and purified as described previously (27). A complex of GLP-1 and the ECD was purified by size exclusion chromatography (supplemental Fig. S1). The purified complex was characterized by SDS-PAGE (supplemental Fig. S1), concentrated, and crystallized by hanging drop vapor diffusion. Diffraction data were collected from a single crystal using the beamline 1911-3 at MAX-lab (Lund, Sweden), and the structure of GLP-1 in complex with the GLP-1R ECD was solved to 2.1 Å resolution by molecular replacement (Fig. 1*A*). We removed exendin-4(9–39) from the structure of the exendin-4(9–39)-ECD complex (Protein Data Bank code 3C59) and used the apo-form of GLP-1R ECD as the search model for the molecular replacement. GLP-1 was then built into the model; its position was unambiguous due to good electron density for most of the ligand. Data collection and refinement statistics are summarized in Table 1. Throughout the text, GLP-1 and exendin-4 residues are designated with * and **, respectively. Exendin-4 is numbered 1–39 and GLP-1 is numbered 7–37, due to post-translational processing. The aligned sequences are illustrated in Fig. 1*B*.

Structure of the GLP-1R Extracellular Domain—The crystal structure of the ECD in the GLP-1-bound form shown here is very similar to the exendin-4(9–39)-bound form shown previously (root mean square deviation of 0.79 Å for C^{α} atoms of the ECD) (18). The ligand-binding sites are identical, which is not surprising given the competitive binding of GLP-1 and exendin-4 for the full-length GLP-1R as well as the isolated ECD (27). However, we have previously shown that divergent residues in the two ligands are responsible for their different physical properties in solution and their different affinity for the ECD (27). As described below, these divergent residues give rise to structural differences in the two ligand-bound forms of the ECD at the level of specific side chain conformations.

TABLE 1
Data collection and refinement statistics
The data set was collected from a single crystal. Values in parentheses are for the highest resolution shell (2.2-2.1 Å).

Space group	$P2_122_1$
Unit cell dimensions (Å)	
A	35.7
B	42.7
C	95.1
Data collection	
Wavelength	1.0 Å
Resolution range	95.1–2.1 Å (2.2–2.1 Å)
Total reflections	73,811
Unique reflections	10,348
Completeness	97.9%
$I/\sigma(I)$	14.0 (5.3)
R_{sym}	11.1 (44.9)
Refinement statistics	
No. of non-hydrogen atoms	1109
Resolution	95.1-2.1 Å (2.155-2.100 Å)
Total reflections	8786
Reflections in test set	429
R_{work}	0.181 (0.187)
R_{free}	0.226 (0.255)
Average *B*-factors	13.6
Root mean square deviation	
Bond lengths	0.02 Å
Bond angles	1.7°

Structure of GLP-1 and Its Interactions with the Extracellular Domain of GLP-1R—GLP-1 is a continuous α-helix from Thr^{13*} to Val^{33*}, with a kink around Gly^{22*}. The residues between Ala^{24*} and Val^{33*} interact with the ECD (Fig. 1, *A* and *B*). The amphiphilic nature of this α-helical segment enables hydrophilic and hydrophobic interactions through opposite faces of the α-helix. The hydrophilic face is comprised by residues Gln^{23*}, Lys^{26*}, Glu^{27*}, and Lys^{34*}. Lys^{26*} is the only one of these that may interact directly with the ECD (Fig. 2*A*). The side chain of Lys^{26*} may form a hydrogen bond with the side chain of Glu^{128} (~3.0 Å), but the *B*-factor is rather high for both residues (~25 compared with 10 for well defined residues), suggesting that this is not a strong interaction. In particular, the electron density of the Lys^{26*} side chain is rather weak. In exendin-4(9–39), the amphiphilic character is more pronounced, and the α-helical conformation is further stabilized by intramolecular interactions between Glu^{16**}, Glu^{17**}, Arg^{20**}, Glu^{24**}, and Lys^{27**} on the hydrophilic face (18). In addition, Arg^{20**} and Lys^{27**} interact with Glu^{128} and Glu^{127} of the ECD, respectively (18). The corresponding intramolecular stabilization is not possible in GLP-1 due to a less favorable alignment of oppositely charged residues (Fig. 1*B*), and Glu^{127} of the ECD is not involved in binding of GLP-1 (Fig. 2*A*).

The hydrophobic face of GLP-1, which interacts with the ECD, is defined by Ala^{24*}, Ala^{25*}, Phe^{28*}, Ile^{29*}, Leu^{32*}, and Val^{33*} (Fig. 2*B*). The importance of Phe^{28*}, Ile^{29*}, and Leu^{32*} in GLP-1 binding has been demonstrated previously by Ala scanning of GLP-1 (38). Substitution of Phe^{28*} with Ala had the most severe effect on GLP-1 affinity in the Ala scan (IC_{50} value increased by 1300-fold), and indeed Phe^{28*} is centrally positioned in the ligand-receptor interface emphasizing the importance of this hydrophobic ligand-receptor interaction. The I29*A and L32*A substitutions also reduced GLP-1 affinity significantly (IC_{50} value increased by 93- and 17-fold, respectively) (38). Trp^{31*} is also on the hydrophobic face of GLP-1 but is rather solvent-exposed and does not interact with the ECD (Fig. 2*B*). Trp^{31*} is conserved in the glucagon peptide family (GLP-1, exendin-4, glucagon, GIP, and GLP-2, see Fig. 1*B*), which implies a unique role of this residue. However, substitution of Trp^{31*} with Ala only reduced the binding affinity of GLP-1 slightly at the full-length GLP-1R (38), so the role of Trp^{31*} in receptor binding is unclear.

Val^{33*} is the final residue in the α-helix of GLP-1, and it is the final residue in the C terminus of GLP-1 that interacts with the ECD (Fig. 2, *A* and *C*). The side chain of Val^{33*} makes hydrophobic contacts with Tyr^{69} and Leu^{123}, and the backbone carbonyl of Val^{33*} interacts through a hydrogen bond with one of the terminal nitrogens of Arg^{121} (Fig. 2, *A* and *C*). The other terminal nitrogen of Arg^{121} interacts with a water mol-

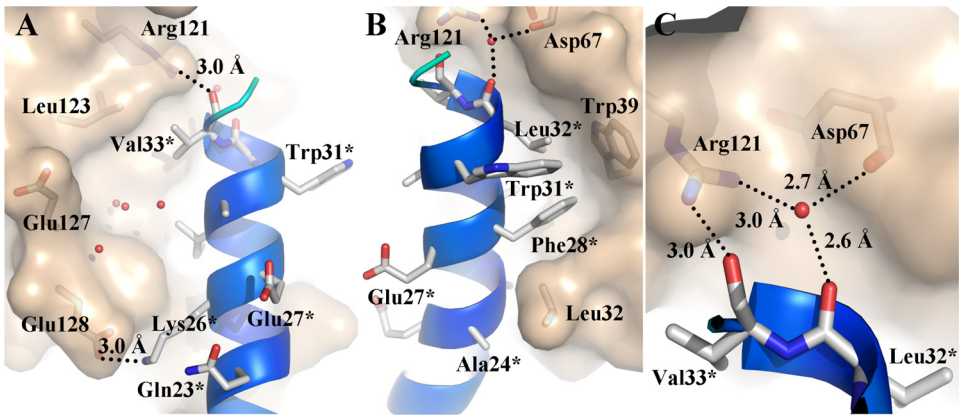

FIGURE 2. **Interactions between GLP-1 and GLP-1R ECD.** *A, ribbon* diagram of GLP-1 and its hydrophilic interactions with GLP-1R ECD. GLP-1 is colored in *cyan*, and residues Gln^{23*}, Lys^{26*}, Glu^{27*}, Trp^{31*}, and Val^{33*} are illustrated as *sticks*. Receptor residues Arg^{121}, Leu^{123}, Glu^{127}, and Glu^{128} are shown as *sticks*. The surface of the hydrophilic binding cavity of ECD is illustrated in *gray*. *B,* ribbon diagram of GLP-1 and its hydrophobic interactions with GLP-1R ECD. GLP-1 residues Ala^{24*}, Glu^{27*}, Phe^{28*}, Trp^{31*}, and Leu^{32*} are illustrated as *sticks*, and so are ECD residues Leu^{32}, Trp^{39}, Asp^{67}, and Arg^{121}. The surface of the hydrophobic binding cavity of ECD is illustrated in *gray*. *C,* ribbon diagram illustrating a common motif found in the GLP-1R ECD and in the GIP receptor ECD. The side chain of Arg^{121} interacts with the backbone carbonyls of Asp^{67} and Leu^{32*} through a water molecule. GLP-1 residues Leu^{32*} and Val^{33*} are illustrated as *sticks*, and so are ECD residues Asp^{67} and Arg^{121}.

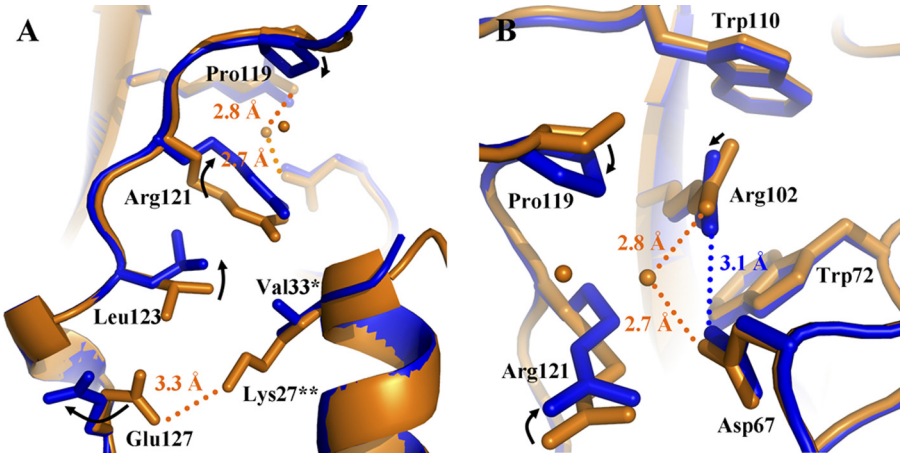

FIGURE 3. **Differences between the GLP-1- and exendin-4(9–39)-bound structure of ECD.** *Ribbon* diagrams showing significant differences in side chain conformations between the GLP-1-bound structure and the exendin-4(9–39)-bound structure of GLP-1R ECD. Receptor and ligand residues are highlighted in *blue* for the GLP-1-bound structure and in *orange* for the exendin-4(9–39)-bound structure. Water molecules in *orange* are present only in the exendin-4(9–39)-bound structure. *A*, one diverging residue, Val^{33*} of GLP-1 and Lys^{27**} of exendin-4(9–39), causes a shift in the conformations of four residues, namely Glu^{127}, Leu^{123}, Arg^{121}, and Pro^{119}. *B*, GLP-1-specific conformations affect the conserved core of the ECD by rotating the guanidine group of Arg^{102} and by decreasing the distance between Asp^{67} and Arg^{102} compared with the exendin-4(9–39)-bound structure without affecting the relative position of Trp^{72} and Trp^{110}.

ecule, which is also coordinated by the backbone carbonyl groups of Asp^{67} and Leu^{32*} (Fig. 2*C*). A water molecule is coordinated by the same residues in the exendin-4(9–39)-bound ECD, and Arg^{121} interacts with the backbone carbonyl of Lys^{27**} of exendin-4(9–39) in a manner similar to the interaction with Val^{33*} of GLP-1. Even though exendin-4 has a C-terminal nine-residue extension, the so-called Trp cage, the extent of the α-helix is similar for GLP-1 and exendin-4, and this particular length of the α-helix fits nicely into the ECD binding pocket.

Judging from the two ligand-bound ECD structures, the hydrophobic ligand-receptor interface is highly conserved, and the C-terminal α-helical structure of the ligands ends at the same position with a similar arrangement of receptor interactions mediated by the backbone of the ligand. Hence, the differential affinity for the ECD may best be explained by the lower α-helical propensity of GLP-1 in solution and by weaker receptor interactions compared with exendin-4, as suggested previously (27).

Unique Structural Features of the GLP-1-bound Extracellular Domain—The divergent residues in GLP-1 and exendin-4 are not only responsible for the different physical properties of the ligands in solution and their differential affinity for the GLP-1R ECD, they also influence the conformation of certain residues in the ECD structures. The crystal structure presented here suggests that one diverging residue in the two ligands (Val^{33*} of GLP-1 and Lys^{27**} of exendin-4(9–39)) causes a shift in the conformations of four residues in (or close to) the binding pocket of the ECD, namely Glu^{127}, Leu^{123}, Arg^{121}, and Pro^{119} (Fig. 3*A*). In exendin-4(9–39), Lys^{27**} interacts with Glu^{127}, and the positioning of the Lys^{27**} side chain appears to be guided by a hydrophobic interaction with Leu^{123}. In GLP-1, Val^{33*} is unable to interact with Glu^{127} causing Glu^{127} to change rotamer conformation and point its side chain away from GLP-1. The side chain of Leu^{123} is flipped toward Arg^{121}, which again is flipped toward Pro^{119}, thereby closing an otherwise water-accessible cavity observed in the exendin-4(9–39)-bound structure (Fig. 3*A*). The closing of this cavity is assisted by a side chain flip of Pro^{119} toward Arg^{121}. The apparent GLP-1-specific conformations affect the conserved core of the ECD by rotating the guanidine group of Arg^{102} and by decreasing the distance between Asp^{67} and Arg^{102} compared with the exendin-4(9–39)-bound structure without affecting the relative position and conformation of Trp^{72} and Trp^{110} (Fig. 3*B*). This enables a direct interaction through a hydrogen bond between Asp^{67} and Arg^{102} unlike what we observed in the exendin-4(9–39)-bound structure, where a water molecule mediated the interaction between Asp^{67} and Arg^{102} (Fig. 3*B*). The functional consequences of the ligand-specific conformational differences are not known.

Site-directed Mutagenesis of the GLP-1R—To link the structural information of the isolated ECD with the binding and functional properties of the full-length receptor, we targeted the ligand-binding site of the ECD by site-directed mutagenesis. The mutants were characterized by their ability to bind GLP-1 and exendin-4, using the agonist ^{125}I-GLP-1 tracer, and by their ability to stimulate cAMP production in response to GLP-1 and exendin-4 (Table 2). The main objective was to search for mutations with differential effect on GLP-1 and exendin-4. We initially focused on Glu^{127}, which showed an obvious conformational difference in the two ligand-bound structures (Fig. 3*A*), Glu^{127} interacts directly with exendin-4(9–39) but not with GLP-1 (18). The ECD structures suggest that the hydrogen-bonding potential of Glu^{127} is important for exendin-4 binding but not for binding of GLP-1, and this is supported by the site-directed mutagenesis data (Table 2). Mutation of Glu^{127} to Ala reduced the affinity for exendin-4 but not for GLP-1 (6.8- *versus* 1.7-fold, respectively). Glu^{128} interacts with a positively charged residue in both GLP-1 and exendin-4, which may explain why the E128A substitution did not have a differential effect on the binding affinity of GLP-1 and exendin-4. The differential effect of the E127A mutation on GLP-1 and exendin-4 binding is rather small compared with the differential affinity of the isolated ECD shown previously (27). Clearly, the superior helical propensity of exendin-4 contributes strongly to its high affinity for the ECD.

Several interactions are conserved in the two ECD structures, and mutagenesis of the implicated receptor residues was not expected to have differential effects on ligand binding (Table 2). Surprisingly, the L32A mutation reduced both the affinity and potency of exendin-4 relative to GLP-1 (7.1- and 9.5-fold, respectively, see Table 2 and Fig. 4), demonstrating a ligand-specific effect of the L32A mutation. Neither the potency nor

TABLE 2

Functional and binding experiments with GLP-1R mutants

EC_{50} and IC_{50} values are given in pM and nM, respectively, and expression levels are given in fmol/mg total protein. Data represent the mean ± S.E. of three or more independent experiments performed in duplicate. The EC_{50} or IC_{50} values of GLP-1 and exendin-4 are compared with the wild-type GLP-1R using the unpaired *t* test. Numbers in parentheses equal the relative difference between EC_{50} or IC_{50} values of GLP-1 and exendin-4 at each mutant compared with the wild-type receptor. ND means not detectable, possibly due to no expression or no binding. * indicates $p < 0.05$; **, $p < 0.01$; and no asterisk means no significant difference.

Mutant	EC_{50}		IC_{50}		Expression level
	GLP-1	Exendin-4	GLP-1	Exendin-4	
	pM		*nM*		*fmol/mg*
Wild type	11 ± 3.2	5.5 ± 1.7	1.0 ± 0.20	0.76 ± 0.19	5.5 ± 0.16
L32A	12 ± 2.7 (1.1)	52 ± 24 (9.5)	1.1 ± 0.17 (1.1)	5.4 ± 1.5* (7.1)	6.0 ± 0.40
T35A	30 ± 7.4 (2.7)	23 ± 12 (4.2)	3.1 ± 0.80 (3.1)	0.44 ± 0.1** (0.6)	0.38 ± 0.01
V36A	57 ± 32 (5.2)	36 ± 20 (6.5)	2.8 ± 0.86 (2.8)	0.98 ± 0.34 (1.3)	5.7 ± 0.28
E68A	15 ± 2.4 (1.4)	9.5 ± 4.2 (1.7)	1.9 ± 0.90 (1.9)	0.48 ± 0.07 (0.6)	5.7 ± 0.07
Y69A	ND	ND	ND	ND	ND
Y88A	ND	ND	ND	ND	ND
L89A	ND	ND	ND	ND	ND
P90A	55 ± 12* (5.0)	30 ± 7.3* (5.5)	2.8 ± 1.6 (2.8)	1.6 ± 0.28 (2.1)	5.6 ± 0.22
R121A	51 ± 13* (4.6)	44 ± 10* (8.0)	2.3 ± 1.1 (2.3)	1.2 ± 0.02 (1.6)	5.7 ± 0.37
L123A	17 ± 4.6 (1.5)	9.5 ± 1.9 (1.7)	1.1 ± 0.38 (1.1)	0.33 ± 0.04 (0.4)	2.6 ± 0.09
E127A	13 ± 4.2 (1.2)	11 ± 0.45* (2.0)	1.7 ± 0.76 (1.7)	5.2 ± 2.0 (6.8)	5.6 ± 0.46
E127Q	12 ± 3.3 (1.1)	6.8 ± 1.4 (1.2)	1.1 ± 0.27 (1.1)	0.82 ± 0.15 (1.1)	6.11 ± 0.21
E128A	28 ± 6.2 (2.5)	25 ± 6.7* (4.5)	2.7 ± 0.91 (2.7)	1.8 ± 0.48 (2.4)	6.0 ± 0.49
E128Q	10 ± 3.0 (0.9)	8.0 ± 3.3 (1.5)	0.66 ± 0.11 (0.7)	0.45 ± 0.1 (0.6)	5.2 ± 0.12

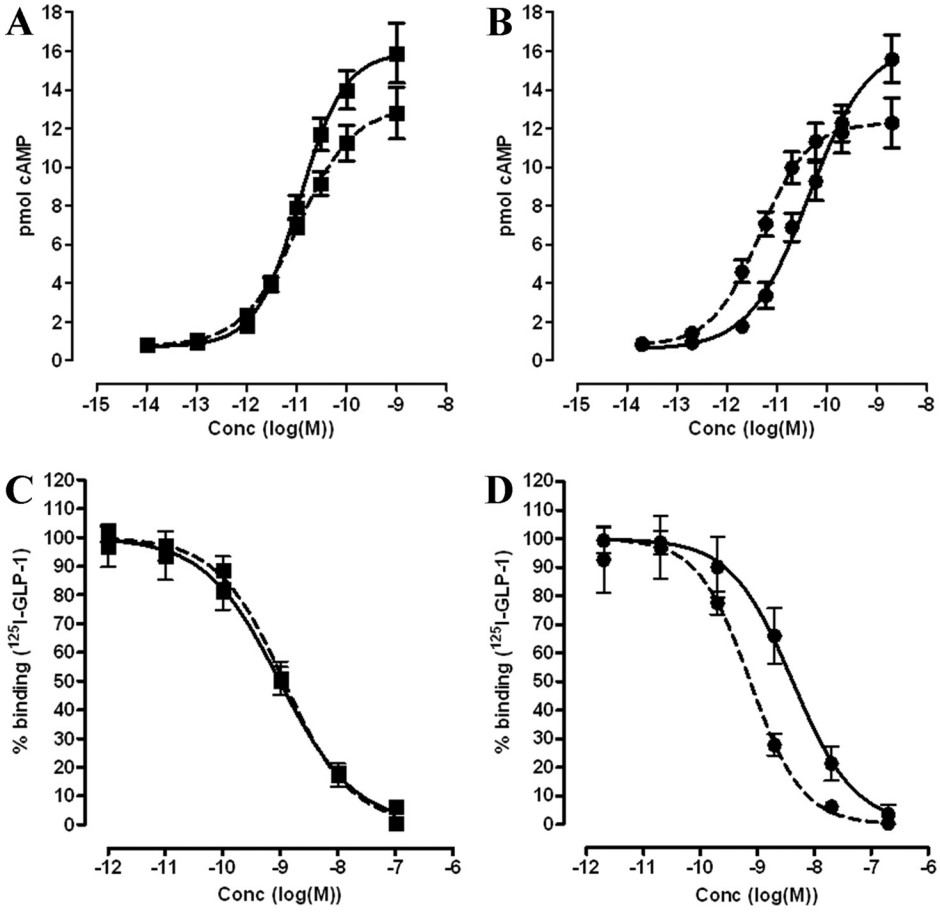

FIGURE 4. **Functional and binding properties of the L32A GLP-1R mutant.** *Upper panel*, stimulation of cAMP production by transiently transfected HEK293 cells expressing the L32A mutant by GLP-1 (*squares, A*) and exendin-4 (*circles, B*). *Dashed* dose-response curves represent cAMP production by GLP-1 and exendin-4 at the wild-type GLP-1R, respectively. *Lower panel,* competition binding assay on plasma membranes from transiently transfected HEK293 cells expressing the L32A mutant. GLP-1 binding curves are presented with squares (*C*) and exendin-4 curves with *circles* (*D*). *Dashed* binding curves represent ^{125}I-GLP-1 displacement by GLP-1 and exendin-4 at the wild-type GLP-1R. Data are normalized according to ^{125}I-GLP-1 binding and correspond to three independent experiments performed in duplicate.

the affinity of GLP-1 was affected by the L32A mutation (Fig. 4), and the expression level of the receptor was similar to wild-type GLP-1R, which confirmed the structural integrity of this receptor mutant. Leu^{32} is the first residue in the α-helix of the GLP-1R ECD, and it defines the border of the hydrophobic binding cavity by interacting with Ala^{24*}, Ala^{25*}, and Phe^{28*} (Fig. 2*B*). The results suggest that Leu^{32} is important for the binding of exendin-4 but not for the binding of GLP-1 to GLP-1R. It is difficult to give a structural explanation of the ligand-specific effect of the L32A mutation by comparing the two ligand-bound forms of the ECD, because the structural differences in this region are quite subtle. Nevertheless, the ligand-specific effect of the L32A mutation supports the existence of differences in the binding modes of GLP-1 and exendin-4 to the full-length GLP-1R. Clearly, the two-domain binding mechanism of the full-length GLP-1R is more complex than binding of the isolated ECD.

Conformation of GLP-1, Receptor-bound and in Solution—GLP-1 is highly flexible in aqueous buffers, whereas in trifluoroethanol a single-stranded α-helix forms (Thr^{13*} to Lys^{34*}) with a less defined α-helical region around Gly^{22*}, as demonstrated by NMR spectroscopy (33, 39). Structure-activity studies of GLP-1 showed that side chain to side chain cyclization by lactam bridge formation of residues 16–20 and 18–22 were well tolerated. Cyclization of residues 11–15 improved potency for GLP-1R compared with the linear coun-

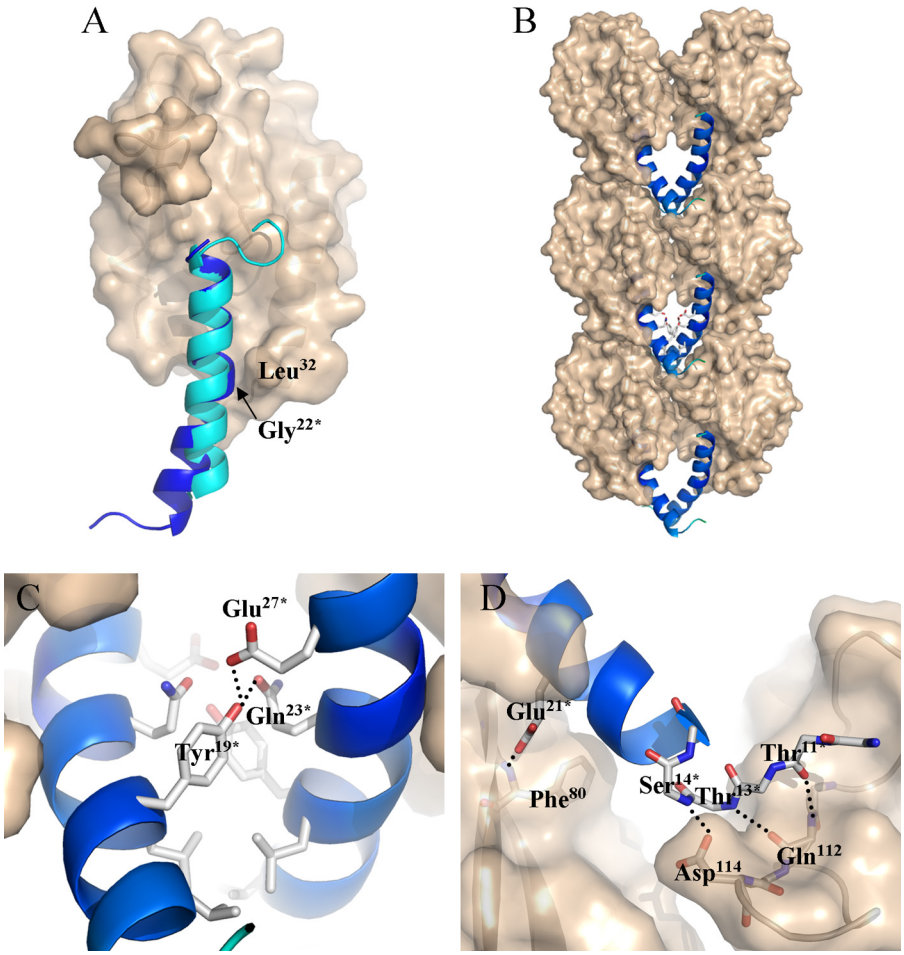

FIGURE 5. **Crystal packing.** *A,* superposition of ECD-bound GLP-1 (*blue*) and exendin-4(9–39) (*cyan*). GLP-1 residue Gly^{22*} denotes a kink in the α-helix, which is situated in close proximity to Leu^{32} of the ECD. *B,* crystal packing involving symmetry-related complex molecules. *C, ribbon* diagram of GLP-1 (*blue*) and its interactions with the ligand of a symmetry-related molecule. Residues Tyr^{19*}, Gln^{23*}, and Glu^{27*} are shown as *sticks*, and the surface of the GLP-1R ECD is shown in *gray*. The packing of complex molecules allows Tyr^{19*} to interact with Gln^{23*} (3 Å) and Glu^{27*} (2.5 Å) in a symmetry-related ligand molecule. *D,* interactions between GLP-1 and residues of symmetry-related ECD molecules. GLP-1 residues Thr^{11*}, Thr^{13*}, Ser^{14*}, and Glu^{21*} are shown as *sticks*. The backbone carbonyl of Thr^{11*} could form a weak hydrogen bond (3.2 Å) to the backbone amide of Gln^{112}, the backbone amide of Thr^{13*} could form a hydrogen bond (2.9 Å) to the backbone carbonyl of Gln^{112}, and the backbone amide of Ser^{14*} may form a hydrogen bond (3 Å) to the side chain of Asp^{114}. The side chain of Glu^{21*} forms a hydrogen bond to the backbone amide of Phe^{80} (2.6 Å).

terpart (40, 41). These data support the existence of an α-helical conformation in the N-terminal part of GLP-1 when bound to the full-length GLP-1R. It is interesting that in the ECD-bound structure, the Thr^{13*}–Glu^{21*} segment of GLP-1 adopts an α-helical conformation even though this segment does not interact with the ECD. The C-terminal segment of GLP-1 (Ala^{24*}–Val^{33*}) is stabilized in a specific α-helical conformation through binding to the ECD. This may subsequently stabilize an α-helical conformation in the N-terminal part of the ligand (Thr^{13*}–Glu^{21*}). This hypothesis is supported by the solution structure of GLP-1 in different concentrations of trifluoroethanol (39). GLP-1 is a random coil in pure water, but adding trifluoroethanol enables the C-terminal segment of GLP-1 to adopt an α-helix conformation. The C-terminal α-helix is gradually extended toward the N-terminus of the peptide with increasing concentrations of trifluoroethanol (39), and it seems possible that a similar mechanism is initiated upon binding of GLP-1 to the ECD. It was recently proposed that α-helix formation of the ligand upon binding to the ECD is an important step in the activation of class B GPCRs (23).

As shown in Fig. 5*A*, the α-helix of GLP-1 has a central distortion of the backbone around Gly^{22*}, which is not observed in the exendin-4(9–39)-bound structure. The distortion is also observed in the NMR structures of GLP-1 in solution (39). However, we cannot exclude that the kink observed in the crystal structure is a result of crystal packing between the N-terminal part of GLP-1 (Gly^{10*}–Glu^{21*}) and symmetry-related ECDs rather than a functionally important characteristic of GLP-1 (Fig. 5, *B–D*). Substitution of Gly^{22*} with Ala was previously shown not to affect the functionality or the binding affinity of GLP-1, which suggests that flexibility around Gly^{22*} is not required for binding to or activation of GLP-1R (38). Interestingly, Leu^{32} of the ECD is positioned right next to the kink of GLP-1 (Fig. 5*A*). Thus, we have demonstrated a ligand-specific effect of the L32A mutation and have shown that ECD-bound GLP-1 has a kink right next to Leu^{32}, whereas ECD-bound exendin-4(9–39) is straight. This may be a coincidence, but it is tempting to speculate that there is a connection between the structural difference of the ligands (kinked or straight helix) and the differential effect of the L32A mutant on binding of the ligands.

A three-dimensional model of GLP-1R was recently published (21). From the NMR structure of GLP-1 in trifluoroethanol (Protein Data Bank code 1D0R (39)), it was suggested that GLP-1 might assume one of two forms when bound to GLP-1R, a slightly kinked α-helix or an L-shaped α-helix, and the authors concluded that the L-shaped α-helix conformation of GLP-1 seemed more reasonable (21). The crystal structure presented here is more compatible with the kinked conformation of GLP-1.

The structure of His^{7*}–Gly^{10*} was not determined in this study probably due to the inherent flexibility in this part of GLP-1 and other peptide ligands for class B receptors (33, 39, 42, 43). The only structural evidence showing a unique conformation comes from a structural study of PACAP(1–21) comparing micelle- and receptor-bound states. Residues 1–7 of PACAP(1–21) adopt a specific β-coil structure upon receptor binding followed by an α-helical structure of residues 8–21 (44). The relevance of the receptor-bound PACAP(1–21) structure for GLP-1 in particular is supported by a previous study of

chimeric PACAP/GLP-1 peptides (45). Substituting five residues from the N-terminus of GLP-1 with those of PACAP (three nonconserved, Fig. 1*B*) had no effect on either the affinity or potency for GLP-1R. Thus, on the basis of both sequence homology and structure-activity, GLP-1 would be expected to adopt a similar conformation upon binding to the GLP-1R TM domain.

Conclusion—The crystal structure presented here shows the molecular details of GLP-1 binding to the GLP-1R ECD, an essential step in the two-domain binding mechanism of GLP-1R and class B GPCRs in general. Collectively, results from structural characterization of GLP-1 in solution, structure-activity analyses of GLP-1 analogues, and the crystal structure of GLP-1 bound to the GLP-1R ECD presented here suggest that GLP-1 is a continuous α-helix from Thr^{13*} to Val^{33*} when bound to the full-length GLP-1R. This is important information for the design of peptide therapeutics targeting GLP-1R. GLP-1 and exendin-4 share the same binding site of the GLP-1R ECD, but the ligand-specific effects on the ECD structure and the ligand-specific effects of receptor mutagenesis support the existence of differences in the binding modes of GLP-1 and exendin-4 to the full-length GLP-1R. The nature of these differences as well as the active conformation of peptide agonists (kinked or not) and a better understanding of the two-domain binding mechanism await structural characterization of the full-length GLP-1R.

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Study II – Small molecule mediated activation of GLP-1R

A small part of the thesis work focused on identifying receptor domains important for small molecule-mediated activation of GLP-1R. The results of this work suggested that TM2 and TM7 are involved in small molecule-mediated activation of GLP-1R and specifically Thr^{391} in TM7 seems to play an important role. The work was published in Pharmacology in 2011.

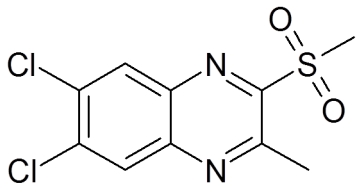

Figure 14. Chemical structure of Compound 3. Compound 3 is structurally related to Compound 2 (Figure 11A), although Compound 3 acts as a partial agonist in stimulating a cAMP response from GLP-1R, whereas Compound 2 acts as a full agonist. The agonistic and allosteric properties of Compound 2 have been described in a number of papers (194;213;231).

Compound 3 is based on a quinoxaline scaffold (Figure 14), which was initially identified in a functional screening of ~ 250.000 compounds (213). In Study II, we found that Compound 3 selectively activated GLP-1R, but not the homologous GCGR. This receptor selectivity of Compound 3 encouraged a chimeric receptor approach to localise the domain of action for Compound 3. The results pointed to a functional importance of the segment defined by TM2 to TM5. Consequently, site-directed mutagenesis was employed to identify specific residues involved in Compound 3 action. The majority of the experimental work was carried out before crystal structures were available for ligand-activated GPCRs. Hence, a three-dimensional model structure of GLP-1R published by Frimurer & Bywater (232) was used to select specific residues for site-directed mutagenesis. According to this model, the side chains of Leu^{192} (TM2), Phe^{195} (TM2) and Leu^{278} (TM4) face the plasma membrane (232), so these three residues were included as control mutations. The model predicts that the side chains of Tyr^{241} (TM3) and Thr^{391} (TM7) point towards residues in other TM helices (232), so Tyr^{241} and Thr^{391} were changed to Ala. We found that Tyr^{241} and Thr^{391} were indeed involved in Compound 3-mediated activation of GLP-1R, as mutation to Ala significantly decreased the potency and efficacy of Compound 3 without affecting GLP-1 potency or affinity (K_d = 0.21 ± 0.02 nM for GLP-1 on wild-type GLP-1R compared to 0.18 ± 0.02 nM and 0.19 ± 0.02 nM on Tyr^{241}-Ala and Thr^{391}-Ala GLP-1R, respectively). Interestingly, two new model structures of GLP-1R have recently been published. The structure published by Coopman *et al.* predicts that the side chains of Tyr^{241} and Thr^{391} are orientated toward each other in the centre of the helical bundle (179), which is supported by our observations. The other model structure, which was recently published by Kirkpatrick *et al.,* predicts that Thr^{391} interacts directly with His^{1} of exendin-4 (233). Our findings suggest that Thr^{391} is not important for GLP-1 binding to GLP-1R. However, several studies support the existence of different binding modes of GLP-1 and exendin-4 to the TM domain of GLP-1R (63;182;189), so mutation of Thr^{391} may have differential effects on GLP-1 and exendin-4 binding.

The experimental work included in Study II was done mainly by Steffen Reedtz-Runge, who constructed the chimeric- and mutant receptors and performed the functional studies. I performed the saturation binding experiments and wrote the manuscript.

The molecular basis for Compound 2 action

The binding sites of several family B small molecule ligands have been mapped (204-206;215;234), but less is known about the structural basis for their action. Extensive truncation of the calcitonin receptor ECD combined with site-directed mutagenesis showed that residues on the border between the ECD and TM1 are important for small molecule action at this receptor (235). This was the only study that specifically identified functionally critical residues for a small molecule agonist of a family B GPCR, until Study II was published. The agonistic and allosteric properties of Compound 2 have been investigated in more detail than those of Compound 3. Still, the molecular basis for Compound 2 action is unknown. Sexton *et al.* recently showed that ECL2 is important for GLP-1 binding and GLP-1 mediated activation of GLP-1R, but not for Compound 2 (236). A subsequent study of the functional effects of GLP-1R single nucleotide polymorphisms (SNPs) identified a single amino acid substitution $Ser^{333} \rightarrow$ Cys in ICL3 that specifically abolished Compound 2-mediated signalling through G_s without affecting peptide ligand-mediated signalling (237). The molecular basis for this observation was not investigated further, so the binding pocket of Compound 2 and the molecular basis for its action remains to be identified.

PAPER II

TRANSMEMBRANE α-HELIX 2 AND 7 ARE IMPORTANT FOR SMALL MOLECULE-MEDIATED ACTIVATION OF THE GLP-1 RECEPTOR

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Transmembrane α-Helix 2 and 7 Are Important for Small Molecule-Mediated Activation of the GLP-1 Receptor

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Key Words
G-protein coupled receptor · Small molecule agonist · cAMP · Mutagenesis

Abstract
Glucagon-like peptide-1 (GLP-1) activates the GLP-1 receptor (GLP-1R), which belongs to family B of the G-protein-coupled receptors. We previously identified a selective small molecule ligand, compound 2, that acted as a full agonist and allosteric modulator of GLP-1R. In this study, the structurally related small molecule, compound 3, stimulated cAMP production from GLP-1R, but not from the homologous glucagon receptor (GluR). The receptor selectivity encouraged a chimeric receptor approach to identify domains important for compound 3-mediated activation of GLP-1R. A subsegment of the GLP-1R transmembrane domain containing TM2 to TM5 was sufficient to transfer compound 3 responsiveness to GluR. Therefore, divergent residues in this subsegment of GLP-1R and GluR are responsible for the receptor selectivity of compound 3. Functional analyses of other chimeric receptors suggested that the existence of a helix-helix interface between TM1 and TM7 is important for the compound 3 response. Furthermore, site-directed mutagenesis revealed that a Phe195-Leu substitution in TM2 and a Thr391-Ala substitution in TM7 increased and decreased the efficacy of compound 3 without disturbing the potency or efficacy of GLP-1. Collectively, differential effects of receptor mutations suggest that TM2 and/or TM7 are important for compound 3-mediated activation of GLP-1R.

Introduction

Glucagon-like peptide 1 (GLP-1) binds and activates the GLP-1 receptor (GLP-1R) on pancreatic β-cells and potentiates insulin secretion in a glucose-dependent manner [1–3]. Given its regulatory function in glucose homeostasis, GLP-1 has an obvious potential in the treatment of type 2 diabetes and obesity. However, GLP-1 is quickly degraded in circulation by dipeptidyl peptidase-

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IV (DPPIV). One approach to circumvent the short half-life of GLP-1 in plasma is to make albumin binding analogues like liraglutide. Liraglutide is an acylated GLP-1 analogue with long-acting biological activity, which possesses all the beneficial effects of native GLP-1 [4, 5]. Peptide-based GLP-1 analogues like liraglutide are administered by subcutaneous injection, but prospective development of orally active pharmaceuticals has driven the search for small molecule agonists that target GLP-1R.

GLP-1 acts through a G-protein-coupled receptor (GPCR). GPCRs can be divided phylogenetically into five main families; rhodopsin, secretin, glutamate, adhesion and frizzled/taste2 with GLP-1R belonging to the secretin family, also known as family B [6]. This family includes a wide range of receptors for peptide hormones like glucagon, glucagon-like peptide-2 (GLP-2), glucose-dependent insulinotropic polypeptide (GIP), pituitary adenylyl cyclase-activating polypeptide (PACAP), vasoactive intestinal polypeptide (VIP), secretin, calcitonin, corticotrophin-releasing factor (CRF) and parathyroid hormone (PTH) [6]. The most closely related receptors to GLP-1R are receptors for peptides of the glucagon branch (glucagon, GLP-2 and GIP), with the highest identity found between GLP-1R and the glucagon receptor (GluR, 44%).

Structural information of family B GPCRs comes from crystal structures of ligand-bound N-terminal extracellular domains (ECDs) [7–12] and from biochemical studies resulting in a two-domain binding model for peptide ligands of family B GPCRs (reviewed in [13]). The ECD of the receptor interacts with the C-terminal part of the peptide, which in turn directs the N-terminal part of the peptide towards the TM domain. However, the activation site of the TM domain is poorly defined. A pair of polar residues in the second TM helix (TM2) is a conserved feature among family B GPCRs, and they appear to be important for peptide agonist binding and activation of several family B receptors [14–17]. Neighboring Arg and Ser residues make up a polar face in TM2 of GLP-1R, which may provide a surface for interaction with either GLP-1 or another TM helix during receptor activation. The latter is supported by site-directed mutagenesis studies on PTH receptor, which suggest that TM2 could be functionally linked to TM7 during ligand binding and activation [18, 19]. Similarly, a recent study that combined molecular modeling and site-directed mutagenesis of the human VPAC1 receptor, demonstrated that interactions between specific residues in TM2, TM3 and TM7 are important for activation of the receptor [20]. Site-directed mutagenesis of a highly conserved His in the cytoplasmic end of TM2 (His180 in GLP-1R) induced constitutive activity of many family B GPCRs and a point mutation in the cytoplasmic end of TM7-induced constitutive activity of PTH receptor [21–25]. Collectively, these results emphasize TM2 and TM7 as potentially important regions for activation of family B GPCRs.

Potential binding sites have been identified for several small molecule ligands that target family B receptors [26–32]. A single residue in the ECD of the human GLP-1R (Trp33) was shown to be responsible for the species selectivity (human/rat) of a small molecule GLP-1R antagonist [32]. Likewise, the competitive antagonist olcegepant binds to the calcitonin gene-related peptide (CGRP) receptor ECD as demonstrated by X-ray crystallography [33]. However, residues in the extracellular end of TM3 and TM5 may determine receptor-subtype selectivity of a small molecule antagonist of the CRF1 receptor [31]. Site-directed mutagenesis of several residues in the TM domain of the rat GluR identified residues in TM2 and TM3, whose substitution with alanine decreased the binding affinity of a small molecule GluR antagonist, while preserving glucagon binding [26]. Thus, small molecule antagonists of family B GPCRs could bind either the ECD or in the TM domain and thereby compete for peptide agonist binding. On the contrary, small molecule agonists probably have to bind to the TM domain or the extracellular loops (ECLs) in order to activate the receptor. Several small molecule agonists have been identified that activate GLP-1R [27, 30, 34] (fig. 1). Screening of a compound library identified a small molecule that stimulated cAMP production in transfected BHK cells expressing GLP-1R [30]. Subsequent optimization generated a full agonist, compound 2 (fig. 1), that stimulated cAMP production with an EC50 value of 101 $\pm$ 21 nmol/l in transfected BHK cells expressing GLP-1R [30]. In more physiological settings, compound 2 potentiated insulin secretion from isolated mouse pancreatic islets in a glucose-dependent manner, whereas neither GLP-1 nor compound 2 potentiated insulin secretion from islets of GLP-1R knockout mice [30]. Other small molecule agonists, like Boc5 (fig. 1) and compound B (fig. 1), have been identified that mimic the effects of GLP-1 in vitro and in vivo [27, 34]. A three-dimensional model structure of GLP-1R was used to predict the putative binding site of Boc5 to be located near the extracellular end of TM3 and TM4 [35]. However, it is worth noting that the agonistic effect of Boc5, but not the agonistic effect of compound 2 or compound B, was blocked by the selective GLP-1R antagonist exendin-(9–39) [27, 30, 34]. In addition, compound 2 allosterically increased the binding affinity of GLP-1 for GLP-1R [30]. Hence, it appears that Boc5 binds

to the orthosteric binding site, whereas compound 2 and compound B bind to an allosteric site in GLP-1R.

The small molecule compound 3 is structurally related to compound 2 (fig. 1) and it selectively activated the human GLP-1R, but not the homologous human GluR in transfected human embryonic kidney (HEK) 293 cells. Compound 3 acted as a partial agonist of GLP-1R. The receptor selectivity of compound 3 encouraged a chimeric receptor approach to identify domains that are important for compound 3-mediated activation of GLP-1R. Site-directed mutagenesis subsequently identified single residues in TM2, TM3 and TM7 of GLP-1R that specifically influenced the efficacy of compound 3 without affecting GLP-1.

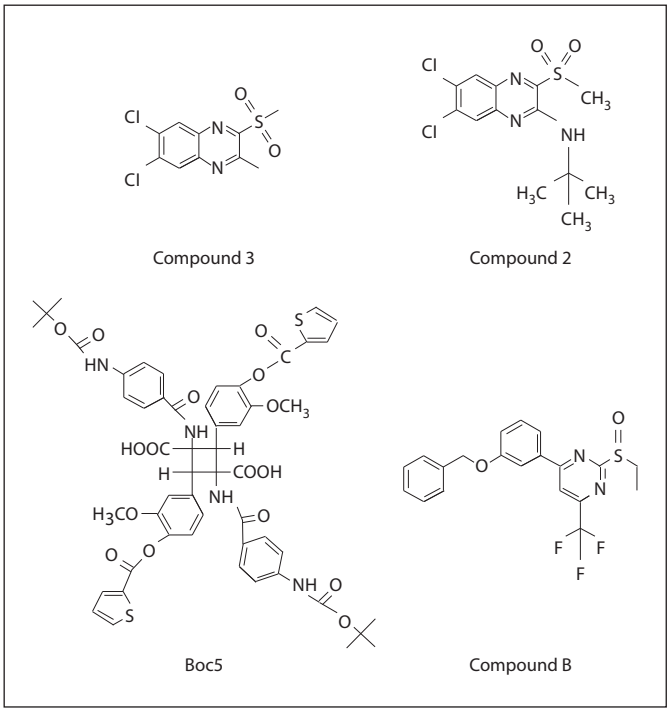

Fig. 1. Structures of small molecule agonists that target GLP-1R. Chemical structures of four GLP-1R agonists. Compound 3 (top left) is structurally related to compound 2 (top right [30]), whereas Boc5 (bottom left [27]) and compound B (bottom right [34]) are based on different scaffolds.

Experimental Procedures

Receptor Constructs

The cDNAs encoding the human GLP-1R and the human GluR were originally obtained from Dr. B. Thorens and Zymogenetics Inc. (Seattle, Wash., USA) and subcloned into the mammalian expression vector pcDNA3.1/V5-His-TOPO® (Invitrogen, Taastrup, Denmark) [36, 37]. The chimeric glucagon/GLP-1 receptors chimera A and chimera B were generated in previous studies [38]. Chimera A was composed of amino acid residues 1–144 of GluR and residues 148–463 of GLP-1R, chimera B was composed of residues 1–169 and 346–477 of GluR and residues 173–347 of GLP-1R. The new chimeric glucagon/GLP-1 receptor chimera C was generated by overlap extension PCR, as previously described and was composed of residues 1–169 of GluR and residues 173–463 of GLP-1R [38]. Site-directed mutagenesis of GLP-1R was done using QuickChange™ (Agilent Technologies, Hoersholm, Denmark) and plasmid DNA was generated and sequenced as previously described [38].

Chemical Synthesis of Peptides and Compound 3

Glucagon, GLP-1 (7–37), ^{125}I-GLP-1(7–36)amide and compound 3 were synthesized and characterized as previously described [30, 38].

Cell Culture and Transient Receptor Expression

HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (90 U/ml and 90 μg/ml, respectively) (Invitrogen). Cells were seeded in T75 flasks, transfected with 9 μg of DNA using the FuGene™ transfection reagent (Roche, Hvidovre, Denmark), harvested 24 h after transfection and used directly in functional experiments or plasma membrane preparations as described previously [38].

Functional Assay

HEK293 cells transiently expressing the desired wild type, mutant or chimeric receptor were resuspended in assay buffer containing 3-isobutyl-1-methylxanthine (IBMX) (Flashplate®, Perkin Elmer, Skovlunde, Denmark) to a cell density of 2×10^6 cells/ml. GLP-1 and glucagon were dissolved and diluted in 0.02% Tween-20, 1% dimethylsufoxide (DMSO).

Compound 3 was initially dissolved in 100% DMSO and diluted to 1% DMSO in 0.02% Tween-20. Cells in assay buffer (50 μl) and GLP-1, glucagon or compound 3 (50 μl) were mixed in 96-well Flashplates (Perkin Elmer), gently agitated for 5 min and incubated for 25 min at room temperature. The resulting intracellular level of cAMP was measured according to supplier's manual and data were analyzed by nonlinear regression analysis using Prism® (GraphPad Software, Inc., La Jolla, Calif., USA). Statistical analyses were performed in Prism using the unpaired t test.

Saturation Binding Assay

Freshly thawed plasma membrane preparations from transiently transfected HEK293 cells expressing wild-type GLP-1R or GLP-1R mutants were pulled through a 25-gauge needle three times and diluted in assay buffer (50 mmol/l HEPES, 5 mmol/l $MgCl_2$, 5 mmol/l EGTA, 0.005 vol% Tween-20, pH 7.4). Membrane preparations ranged between 0.25–2 μg protein/well. Different dilutions of the radioligand ^{125}I-GLP-1(7–36)amide were prepared in assay buffer corresponding to a concentration range of approximately 0.025–0.9 nmol/l. Nonspecific binding was determined with 1 μmol/l GLP-1. Membrane-preparation, GLP-1 and radioligand were mixed in 96-well 0.65-μmol/l filter plates (Millipore, Copenhagen, Denmark) and incubated for 1 h at

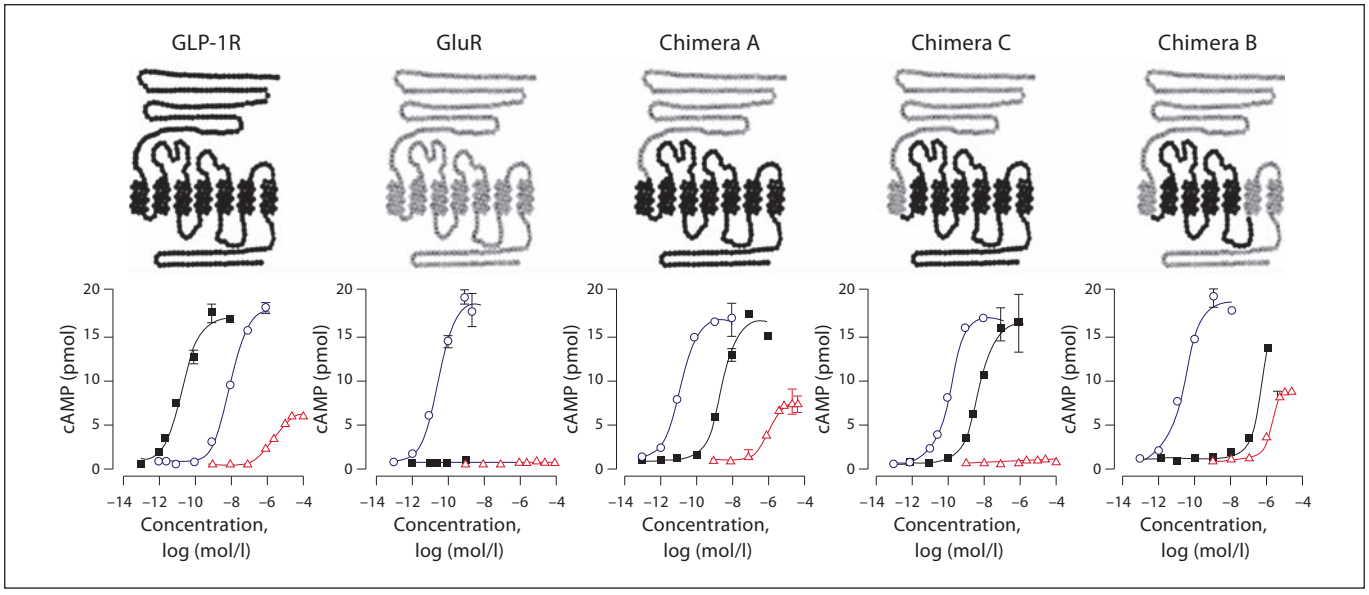

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Fig. 2. Stimulation of cAMP production of transfected HEK293 cells transiently expressing GLP-1R, GluR or chimeric glucagon/GLP-1 receptors by GLP-1, glucagon or compound 3. Each experiment is representative of three or more independent experiments performed in duplicates. GLP-1-induced cAMP stimulation is shown by black curves and squares. Glucagon-induced cAMP stimulation is shown by blue curves and open circles. Compound 3-induced cAMP stimulation is shown by red curves and open triangles. Colors refer to the online version only.

37°C. Subsequently, bound and unbound radioligand were separated by vacuum filtration (Millipore vacuum manifold). The filters were washed twice in 100 μl cold assay buffer and left to dry. Data were fitted as one-site binding using Prism. Statistical analyses were performed in Prism using the unpaired t test.

Results

Glucagon and GLP-1 Receptors

The small molecule compound 3 (fig. 1) stimulated the production of cAMP in GLP-1R-expressing cells with EC_{50} = 1.9 ± 0.11 μmol/l and with 42% efficacy relative to GLP-1 (100%) (fig. 2; table 1). Compound 3 was unable to stimulate cAMP production in GluR-expressing cells (fig. 2). This selectivity for the human GLP-1R encouraged a chimeric receptor approach to identify the domains and residues that are important for compound 3-mediated activation of GLP-1R.

Chimeric Glucagon/GLP-1 Receptors

Chimera A consists of the ECD of GluR and the TM domain of GLP-1R as described previously [38]. The potency of GLP-1 on chimera A was reduced ~100-fold compared to wild-type GLP-1R. However, compound 3 activated chimera A with EC_{50} = 1.6 ± 0.19 μmol/l and with a relative efficacy of 48% (table 1), which is comparable to its potency and efficacy on wild-type GLP-1R. In order to narrow down the TM segments that determine compound 3 activity, compound 3 was analyzed for its ability to activate other more GluR-like chimeric receptors. Chimera A was made more GluR-like by substituting TM1 with that of GluR, and the resulting chimeric receptor, chimera C, was analyzed in functional experiments. Glucagon and GLP-1 activated chimera C with similar intracellular cAMP levels compared to chimera A (fig. 2), and the glucagon/GLP-1 selectivity profile of chimera C was comparable to that of chimera A, although the potency of both peptides was reduced by ~4-fold relative to chimera A. However, compound 3 was almost unable to activate chimera C (fig. 2). The small molecule agonist was analyzed further for its ability to activate chimera B, which is even more GluR-like compared to chimera C. Surprisingly, compound 3 activated chimera B with EC_{50} = 2.4 ± 0.41 μmol/l and an efficacy of 52% relative to glucagon (100%).

Site-Directed Mutagenesis of the GLP-1 Receptor

The functional properties of chimera B indicated that residues within the segment spanning from TM2 to TM5

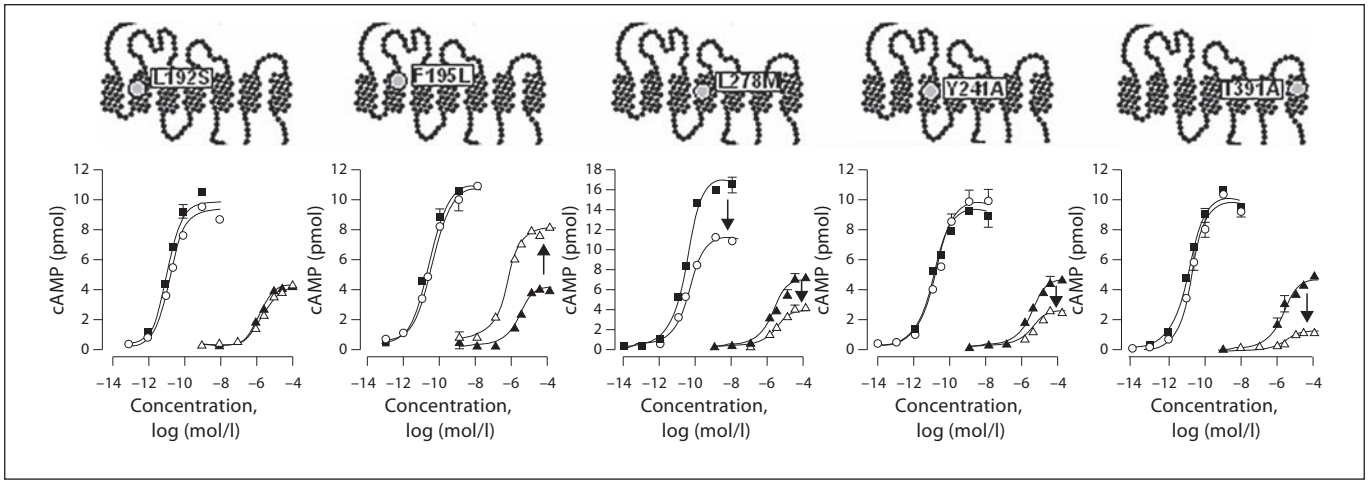

Fig. 3. Stimulation of cAMP production of transfected HEK293 cells transiently expressing GLP-1R or GLP-1R point mutants by GLP-1 or compound 3. All GLP-1R point mutants (illustrated in upper panel) were analyzed in parallel with GLP-1R and each experiment is representative of three or more independent experiments performed in duplicates. cAMP dose-response curves of GLP-1R – GLP-1: closed squares; compound 3: closed triangles. cAMP dose-response curves of GLP-1R point mutants – GLP-1: open circles; compound 3: open triangles.

Table 1. Functional properties of GLP-1R, GluR, chimeric glucagon/GLP-1 receptors and GLP-1R point mutants

Receptor	EC_{50} values			Relative, efficacy[a], %	B_{max}, fmol/mg
	GLP-1, pmol/l	glucagon, pmol/l	compound 3, μmol/l		
GLP-1R	21 ± 2.1	21,000 ± 4,600**	1.9 ± 0.11	42 ± 1.7	275 ± 36
GluR	n.a.	27 ± 6.3	n.a.	n.a.	–
Chimera A	2,000 ± 880**	23 ± 3.5	1.6 ± 0.19	48 ± 2.0	–
Chimera C	7,500 ± 2,200**	110 ± 17**	n.a.	n.a.	–
Chimera B	≥100,000**	40 ± 5.7	2.4 ± 0.41	52 ± 3.0[b, **]	–
Leu192-Ser	19 ± 1.4	–	2.1 ± 0.50	44 ± 5.0	–
Phe195-Leu	19 ± 4.3	–	0.67 ± 0.09***	72 ± 2.4***	163 ± 7.0*
Tyr241-Ala	33 ± 7.4	–	4.2 ± 0.61***	25 ± 1.5***	116 ± 7.0*
Leu278-Met	26 ± 7.5	–	1.8 ± 0.35	37 ± 2.4	–
Thr391-Ala	26 ± 9.7	–	6.3 ± 1.7***	15 ± 1.1***	482 ± 54*

All values represent mean ± SEM of three or more independent experiments performed in duplicates.

[a] The relative efficacy is the maximal response of compound 3 relative to the maximal response of GLP-1 (100%). [b] The relative efficacy is the maximal response of compound 3 relative to glucagon. B_{max} values were measured for GLP-1 only. The EC_{50} values, relative efficacy and B_{max} values of GLP-1, glucagon and/or compound 3 at each chimera or receptor mutant were compared to those of the wild-type receptor using the unpaired t test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. n.a. = No activation (no measurable cAMP level).

of GLP-1R are involved in compound 3-mediated activation. In our initial effort to identify single residues important for compound 3-mediated activation and/or receptor selectivity, we mutated a few residues in TM2 and TM4 based on the three-dimensional model structure of GLP-1R published by Frimurer and Bywater [39]. Three hydrophobic residues, Leu192 (TM2), Phe195 (TM2) and Leu278 (TM4) were substituted with the corresponding Ser, Leu and Met of GluR, respectively (fig. 3). Two additional residues, Tyr241 (TM3) and Thr391 (TM7), were substituted with Ala based on the three-dimensional model structure of GLP-1R [39]. GLP-1 and compound 3 were analyzed in parallel for their ability to stimulate cAMP production in transfected HEK293 cells transiently expressing GLP-1R

or GLP-1R point mutants (fig. 3). The apparent potency of GLP-1 and compound 3 and the efficacy of compound 3 relative to GLP-1 are given in table 1. Wild-type GLP-1R and the Leu192-Ser and Leu278-Met GLP-1R mutants were activated with the same potency and relative efficacy by GLP-1 and compound 3 (table 1). In contrast, the mutations Phe195-Leu, Tyr241-Ala and Thr391-Ala significantly altered the potency and efficacy of compound 3 without disturbing the potency and efficacy of GLP-1 (table 1; fig. 3). The relative efficacy of compound 3 was increased to 72% for the Phe195-Leu GLP-1R mutant, compared to 42% for wild-type GLP-1R ($p < 0.0001$). Similarly, the potency of compound 3 was significantly increased to EC50 = 0.67 ± 0.09 μmol/l($p < 0.0001$) compared to wild-type GLP-1R. The expression level of the Phe195-Leu mutant was slightly decreased compared to wild-type GLP-1R (B_{max} = 163 ± 7.0 fmol/mg compared to B_{max} = 275 ± 36 fmol/mg, p = 0.02; table 1). However, neither the potency nor the efficacy of GLP-1 was affected, which confirmed the structural integrity of the receptor mutant. The Tyr241-Ala and Thr391-Ala mutations significantly decreased the efficacy of compound 3 to 25% (p = 0.0004) and 15% ($p < 0.0001$) relative to GLP-1. Similarly, both mutations significantly decreased compound 3 potency to EC_{50} = 4.2 ± 0.61 μmol/l ($p < 0.0001$) and EC_{50} = 6.3 ± 1.7 μmol/l ($p < 0.0001$), respectively. The expression level of the Tyr241-Ala mutant was slightly decreased compared to wild-type GLP-1R (B_{max} = 116 ± 7.0 fmol/mg compared to B_{max} = 275 ± 36 fmol/mg, p = 0.01; table 1), whereas the expression level of the Thr391-Ala mutant was increased compared to wild-type GLP-1R (B_{max} = 482 ± 54 fmol/mg compared to B_{max} = 275 ± 36 fmol/mg, p = 0.03; table 1).

Discussion

Several small molecule agonists have been identified for GLP-1R [27, 30, 34], yet little is known about their site of interaction with the receptor. We previously identified a selective small molecule ligand, compound 2, that acted as a full agonist and allosteric modulator of GLP-1R [30]. In this study, the structurally related small molecule, compound 3, stimulated cAMP production from GLP-1R, but not from the homologous glucagon receptor (GluR). This selectivity for the human GLP-1R encouraged a chimeric receptor approach to identify the domains and residues that are important for compound 3-mediated activation of GLP-1R.

The GLP-1R ECD determines specificity for the C-terminal part of GLP-1, and it is the primary determinant of the glucagon/GLP-1 selectivity profile of GLP-1R (difference in affinity/potency of glucagon and GLP-1) [38]. In contrast, the GLP-1R TM domain is much less selective and interacts almost equally well with the N-terminal part of GLP-1 and glucagon. Chimera A was constructed for a previous study [38] and consists of the ECD of GluR and the TM domain of GLP-1R. The potency of GLP-1 on chimera A was reduced significantly compared to wild-type GLP-1R, whereas the potency and efficacy of compound 3 on chimera A was comparable to those of wild-type GLP-1R. Based on this result, it seems likely that compound 3 binds to the TM domain of GLP-1R and that the residues responsible for compound 3-mediated activation of GLP-1R are present in the TM domain.

In order to narrow down the TM segments that determine compound 3 activity, compound 3 was analyzed for its ability to activate other more GluR-like chimeric receptors. Chimera C consists of the ECD and TM1 of GluR and TM2-TM7 of GLP-1R. The glucagon/GLP-1 selectivity profile of chimera C was comparable to that of chimera A, but compound 3 was almost unable to activate chimera C. Hence, at this point it seemed that TM1 was very important for compound 3-mediated activation of chimera A. Compound 3 was subsequently analysed for its ability to activate chimera B, which was initially characterized in a previous study [38] and has a stronger glucagon/GLP-1 selectivity profile than chimera A because of additional selectivity determinants in the GluR TM domain. Surprisingly, the negative impact of TM1 of GluR on compound 3-mediated activation of chimera C was rescued by adding TM6 and TM7 of GluR, regardless of the loss of potency of GLP-1 relative to glucagon. The rescue of the compound 3 response of chimera B is probably best explained by the existence of a helix-helix interface between TM1 and TM7. Accordingly, some degree of molecular incompatibility between TM1 and TM7 of chimera C eliminated the compound 3 response while preserving almost entirely the glucagon/GLP-1 selectivity profile and potency. The molecular incompatibility probably destabilized the active conformation obtained by compound 3 and/or directly affected the binding of compound 3. We cannot distinguish between these two possibilities. Nevertheless, the GLP-1R segment of chimera B (TM2 to TM5 and connecting loops) was sufficient to transfer compound 3 responsiveness to GluR and therefore divergent residues within this segment are important for compound 3-mediated activation.

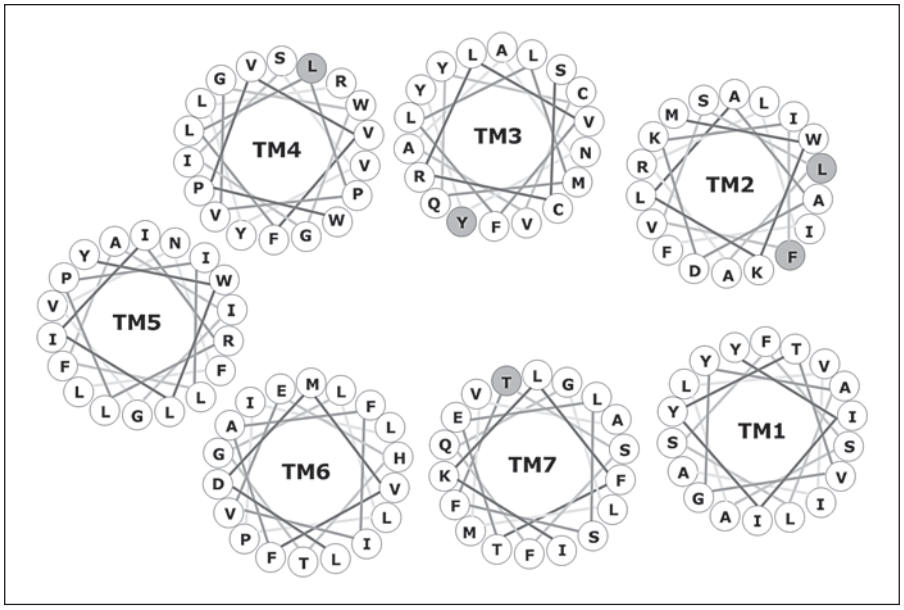

Fig. 4. Helical wheel diagram of GLP-1R. The helical wheel diagram represents the central 18 residues in each helix according to the most recent GLP-1R model [44]. In this study, Leu192 (TM2), Phe195 (TM2) and Leu278 (TM4) (highlighted in grey) were substituted with the corresponding Ser, Leu and Met of GluR, respectively. Tyr241 (TM3) and Thr391 (TM7) (also highlighted in grey) were substituted with Ala.

In the beta-2-adrenergic receptor, a family A GPCR, intramolecular hydrogen bonds link amino acid side chains in TM2 and TM7 [40, 41], in analogy with the functional coupling of TM2 and TM7 of the PTH receptor and the VPAC1 receptor [18–20, 42]. The proximity of TM2 and TM7 of GluR probably accommodates the glucagon N-terminus as shown previously [43] and the rescue of the compound 3 response by chimera B suggests that a functionally important interface exists between TM1 and TM7. Collectively, previous studies combined with our data indicate that TM1, TM2 and TM7 close up the 7TM helical bundle of family B 7TM GPCRs in analogy with the overall arrangement of the 7TM helices of family A GPCRs.

The functional properties of chimera B indicated that residues within TM2-TM5 of GLP-1R are involved in compound 3-mediated activation. Based on the model structure of GLP-1R used to predict the putative binding site of Boc5 [35], it seems likely that residues in the extracellular end of TM2-TM5 are important for the small molecule mediated response. However, it was beyond the scope of this study to systematically investigate all the divergent residues within this region. We mutated a few residues in TM2, TM3, TM4 and TM7 based on the three-dimensional model structure of GLP-1R published by Frimurer and Bywater [39]. According to this model, the side chains of Leu192 (TM2), Phe195 (TM2) and Leu278 (TM4) face the plasma membrane [39], so mutation of these three residues was included as a negative control. The side chains of Tyr241 (TM3, conserved in GLP-1R and GluR) and Thr391 (TM7, Ser389 in GluR) could point towards potential small molecule binding sites [39], so Tyr241 and Thr391 were substituted with Ala. The Thr391-Ala substitution was included to further investigate the importance of TM7 in compound 3-mediated activation of GLP-1R. The Phe195-Leu, Tyr241-Ala and Thr391-Ala mutations significantly altered the potency and efficacy of compound 3 without disturbing the potency and efficacy of GLP-1. This corresponds well with the observation made by Coopman et al. [44] who recently showed that the Thr391-Ala mutation does not affect GLP-1 affinity or potency. Surprisingly, the relative efficacy and potency of compound 3 was increased for the Phe195-Leu mutant compared to wild-type GLP-1R despite a decrease in receptor expression level. However, neither the potency, nor the efficacy of GLP-1 was affected, which confirmed the structural integrity of this receptor mutant. Interestingly, the model structure of GLP-1R used to predict the putative binding site of Boc5 [35] shows that the side chain of Phe195 is positioned near the binding site of Boc5 in the interface between TM2 and TM7. The most recent model structure of GLP-1R predicts a slightly different position of Phe195, i.e. in the interface between TM1 and TM2 [44] (fig. 4). Hence, our results support the existence of a functional interface between TM1, TM2 and TM7 in GLP-1R, and Leu195 may either facilitate conversion between inactive and active receptor conformations or stabilize active conformations

and thereby improve the potency and efficiency of activation by compound 3. Clearly, other unidentified divergent residues in the GLP-1R segment of chimera B determined the receptor selectivity of compound 3.

The Tyr241-Ala and Thr391-Ala mutations significantly decreased the efficacy and potency of compound 3. The expression level of the Tyr241-Ala mutant was slightly decreased compared to wild-type GLP-1R, so we cannot rule out the possibility that the effects of the Tyr241-Ala mutation were caused by reduced receptor expression. However, the expression level of the Thr391-Ala mutant was increased compared to wild-type GLP-1R. It is unlikely that the increase in receptor expression level caused the decrease in compound 3 potency and efficacy, and the structural integrity of the Thr391-Ala mutant was confirmed by the unaffected potency and efficacy of GLP-1R. Therefore, our results suggest that Thr391 and possibly Tyr241 are required for optimal compound 3-mediated activation of GLP-1R. Interestingly, the most recent model structure of GLP-1R predicts that the side chains of Tyr241 and Thr391 are orientated toward each other in the centre of the helical bundle (fig. 4) [44]. Hence, this region may be involved in compound 3-mediated activation of GLP-1R. In this study, Thr391 was in fact targeted by the chimeric receptor approach. In chimera B, it appeared that the corresponding Ser389 of GluR successfully substituted Thr391 of GLP-1R. Hence, it seems that a polar side chain in position 391 (Thr or Ser) is necessary for stabilization of the active conformation obtained with compound 3 or it is necessary for direct interaction with compound 3. We cannot distinguish between these two possibilities based on these experiments. In contrast, the polar side chain of Thr391 is not necessary for GLP-1-mediated activation. It is possible that the active conformations stabilized by GLP-1 and compound 3 are different and/or compound 3 needs Thr391 for binding but GLP-1 does not.

Conclusion

In the present study, we have used a chimeric receptor approach followed by site-directed mutagenesis to identify domains and subsequently specific residues that are important for the compound 3-mediated activation of GLP-1R. Our results suggest that TM2 and TM7 are involved in compound 3-mediated activation of GLP-1R and specifically Thr391 in TM7 seems to play an important role for compound 3 but not for GLP-1. It is believed that TM2 and TM7 are important for peptide agonist binding and activation, suggesting that the same helices (TM2 and TM7) are involved in small molecule and peptide agonist mediated activation.

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Disclosure Statement

The research reported in this article is impartial, and there is no conflict of interest that could prejudice the research.

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Study III – A Cysteine-deprived and C-terminally truncated GLP-1R

In 2002, Rudolph *et al*. developed a method to express, purify and refold the GLP-1R ECD (172). This marked the starting point of a year-long collaboration between Novo Nordisk and the group of Prof. Rainer Rudolph at the Martin Luther University in Halle, Germany. Part of the collaboration focused on expressing the full-length GLP-1R in *E. coli* inclusion bodies as well as developing techniques to solubilise and refold the receptor. The work included in Study III aimed to simplify the refolding process by modifying GLP-1R by site-directed mutagenesis. Several cysteine-deprived and/or C-terminally truncated GLP-1Rs were constructed. Functional characterisation of these receptors combined with binding experiments showed that seven cysteine residues and more than half of the C-terminal tail are not required for GLP-1 binding or function (Figure 15). I undertook all the experimental work included in Study III and wrote the manuscript. The manuscript will be submitted to 'Peptides' in the near future.

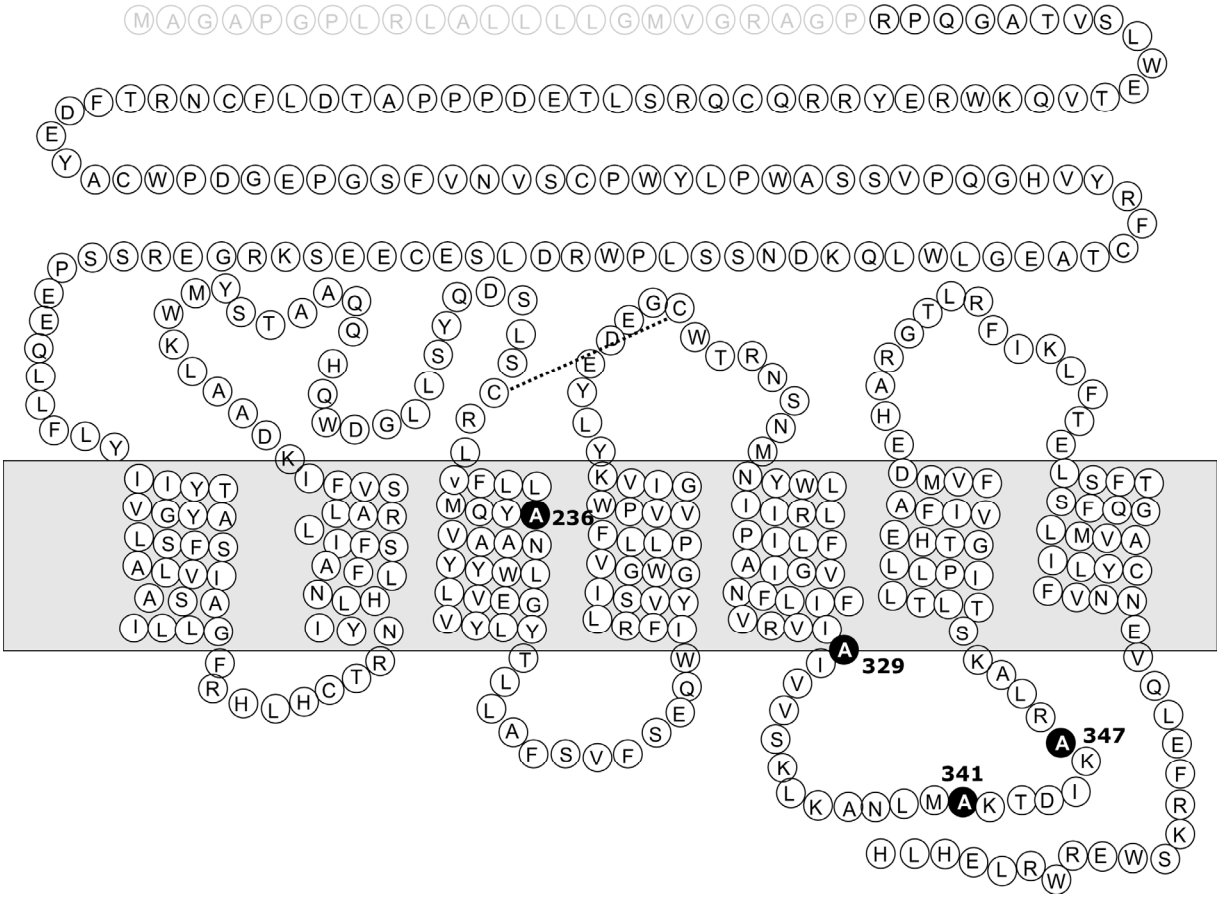

Figure 15. A fully functional cysteine-deprived and C-terminally truncated GLP-1R. Study III describes the development and characterisation of a cysteine-deprived and C-terminally truncated GLP-1R. The 'Cys $1_{1\text{-}426}$' mutant shown above contains four Cys-Ala substitutions (Cys^{236}-Ala, Cys^{329}-Ala, Cys^{341}-Ala and Cys^{347}-Ala) and lacks the last 37 amino acids of the C-terminal tail. The affinity and potency of GLP-1 is similar for 'Cys $1_{1\text{-}426}$' compared to wild-type GLP-1R. Three cysteines in the distal part of the C-terminal tail can also be changed to Ala without compromising GLP-1 function or binding. The putative signal sequence is shown in grey.

Challenges in GPCR crystallography

Until recently, our understanding of GPCR structure was based mainly on the crystal structures of the inactive state of rhodopsin (118;238;239). Rhodopsin is more stable than ligand-activated GPCRs, and it is possible to obtain large quantities of the receptor from natural sources. Other GPCRs are expressed at relatively low levels in native tissues, so efficient expression systems are required to produce large quantities of recombinant receptors. Also, ligand-activated GPCRs contain unstructured regions and cycle between inactive- and active states, which decreases stability even further. In addition, most GPCRs are unstable in detergent, which severely limits the crystallisation conditions. To overcome these changes, all the GPCRs crystallised since rhodopsin have been modified by site-directed mutagenesis. Modifications include thermostabilising mutations (126), truncation of the receptor C-terminal (123;126;130), removed glycosylation sites (130) and mutations that increase the expression level of the receptor (130). In addition, the conformational stability of the GPCRs has been improved by means of ligand binding (122-125;130), T4L fusion (124;125;129;240) and binding of various antibodies (123;128;130). These structural modifications have contributed to the recent advances in GPCR crystallography.

Over the years, *E. coli* has been widely used for expression of recombinant proteins that do not require post-translational modifications (241). However, high expression of recombinant proteins in *E. coli* often leads to accumulation of the protein of interest into insoluble aggregates known as inclusion bodies. Inclusion body proteins are misfolded and devoid of biological activity, so elaborate solubilisation, refolding and purification procedures are required to obtain a fully functional protein (242). During the refolding process, misfolded proteins and protein aggregates can hamper the recovery of correctly folded proteins (242). Formation of random intra- and intermolecular disulphide bonds can lead to misfolding of the protein. Hence, a cysteine-deprived form of the protein of interest is desirable, as it may increase the yield of correctly folded protein.

MANUSCRIPT

DEVELOPMENT OF A CYSTEINE-DEPRIVED AND C-TERMINALLY TRUNCATED GLP-1 RECEPTOR

Development of a Cysteine-deprived and C-terminally Truncated GLP-1 Receptor

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Footnotes:

[1]The abbreviations used are: $\beta_{1/2}$AR, $\beta_{1/2}$-adreneric receptor; CLR, calcitonin receptor-like receptor; CRFR1, type-1 corticotrophin-releasing factor receptor; ECL, extracellular loop; FRET, Fluorescence resonance energy transfer; GCGR, glucagon receptor; GIP-R, glucose-dependent insulinotropic polypeptide receptor; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; GPCR, G protein-coupled receptor; ICL, intracellular loop; PAC1-R, type-1 pituitary adenylate cyclase-activating polypeptide receptor; PTH-1R, type-1 parathyroid hormone receptor; PTHrP, parathyroid hormone related peptide; TM, transmembrane; VIP, vasoactive intestinal polypeptide.

Abstract

The glucagon-like peptide-1 receptor (GLP-1R) belongs to family B of the G-protein coupled receptors (GPCRs), and has become a promising target for the treatment of type 2 diabetes. Here we describe the development and characterization of a fully functional cysteine-deprived and C-terminally truncated GLP-1R. Single cysteines were initially substituted with alanine, and functionally redundant cysteines were subsequently changed simultaneously. The results indicate that Cys^{174}, Cys^{226}, Cys^{296} and Cys^{403} are important for the GLP-1-mediated response, whereas Cys^{236}, Cys^{329}, Cys^{341}, Cys^{347}, Cys^{438}, Cys^{458} and Cys^{462} are not. Extensive deletions were made in the C-terminal tail of GLP-1R in order to determine the limit for truncation. We observed a direct correlation between the length of the C-terminal tail and specific binding of ^{125}I-GLP-1, indicating that the membrane proximal part of the C-terminal is involved in receptor expression at the cell surface. The results show for the first time that seven cysteines and more than half of the C-terminal tail can be removed from GLP-1R simultaneously without compromising GLP-1 binding or function.

1. Introduction

Glucagon-like peptide-1 (GLP-1)[1] is a peptide hormone, which is released from intestinal L-cells in response to food intake [1;2]. GLP-1 stimulates the synthesis and release of insulin from pancreatic β-cells in a glucose-dependent manner [3]. In addition, GLP-1 has a number of physiological effects that contribute to reducing blood sugar and body weight including inhibition of glucagon secretion [4;5] and gastric emptying [6;7], and reduction of food intake [8-10]. Hence, GLP-1-based therapies are becoming increasingly attractive for the treatment of type 2 diabetes. The actions of GLP-1 are mediated through the GLP-1 receptor (GLP-1R), which is a G protein-coupled receptor (GPCR). The human GLP-1R was first cloned in 1992 and belongs to family B of the GPCRs [11]. GPCRs belonging to family B are distinguished by their large extracellular N-terminal domain (ECD) which is important for ligand binding and selectivity [12;13]. The structure of the isolated ECD has been solved for several family B GPCRs with or without receptor-bound ligand. The structures include the ECD of type-1 corticotrophin-releasing factor receptor (CRFR1) [14], the type-1 pituitary adenylate cyclase-activating polypeptide receptor (PAC1-R) [15], the glucose-dependent insulinotropic polypeptide receptor (GIP-R) [16], GLP-1R [17;18], the glucagon receptor (GCGR) [19] and the type-1 parathyroid hormone receptor (PTH-1R) [20].

The tertiary structure of GLP-1R ECD is stabilized by three conserved disulfide bonds and by several intramolecular interactions [17]. Hence, the six cysteines within the ECD are essential for the structure and function of GLP-1R. The GLP-1R contains a total of seventeen cysteine residues of which six reside in the ECD. The remaining eleven cysteines are found in the transmembrane (TM) helices, the loops and in the intracellular C-terminal tail (Figure 1). The

function and importance of some of these cysteines has been investigated by site-directed mutagenesis. For instance, it was recently shown that Cys^{226} in the first extracellular loop (ECL1) most likely forms a disulfide bond with Cys^{296} in ECL2 of GLP-1R. A disulfide bond has been located in the same topological position in several family A GPCRs, where it seems to be important for cell surface expression and ligand binding [21-23]. The existence of a disulfide bond between cysteines in ECL1/TM3 and ECL2 has also been proposed for other family B GPCRs including the vasoactive intestinal polypeptide (VIP) receptor [24] and the PTH-1R [25]. Substitution of Cys^{174} with Ala in the first intracellular loop (ICL1) of GLP-1R has been shown to reduce GLP-1 efficacy and receptor expression levels [26]. Similarly, a Cys^{438}-Ala substitution in the C-terminal tail of GLP-1R significantly reduced GLP-1 efficacy although the expression level of the receptor was increased [27]. Of all the Cys-Ala substitutions reported for GLP-1R, only a Cys^{329}-Ala mutation in TM5/ICL3 showed no reduction in receptor expression level or GLP-1-mediated response [26]. Hence, Cys^{329} is currently the only redundant cysteine residue reported for GLP-1R.

It has been shown for several family A GPCRs that truncation of the C-terminal tail can improve receptor stability. A combination of C-terminal truncation and other sequence modifications have been applied to family A GPCRs, which ultimately facilitated crystallization of the TM domain of these receptors [28-30]. Fluorescence resonance energy transfer (FRET) based studies have shown that the C-terminal tail and ICL3 are the most unstructured regions of the β_2-adreneric receptor (β_2AR) [31]. Hence, the last 48 amino acids were removed from the C-terminal of β_2AR, and two different approaches were employed to stabilize ICL3 in the first crystal structures of the inactive conformation of this receptor [28;32]. Similarly, more than 100 amino acids were removed from the C-terminal tail of the turkey β_1-adreneric receptor (β_1AR) and several mutations and deletions were introduced to facilitate crystallization of this receptor in complex with the high affinity antagonist cyanopindolol [29]. Preceding studies had shown that C-terminal truncation of β_1AR increased receptor expression levels in Sf9 insect cells as well as HEK293 mammalian cells, and the truncated receptors were readily solubilized from the membrane compared to the wild-type receptor [33]. Such comprehensive studies of the C-terminal tail have not been reported for GLP-1R. However, truncation experiments indicate that the distal part of GLP-1R C-terminal is not involved in the signal transduction process [34], and similar observations have been made for other family B GPCRs [35;36]. Instead, phosphorylation of three serine doublets in the distal part of the C-terminal (Ser^{441}/Ser^{442}, Ser^{444}/Ser^{445} and Ser^{451}/Ser^{452}) appears to be important for desensitisation and internalisation of GLP-1R [37].

The aim of the work described in this paper was to develop a fully functional cysteine-deprived and C-terminally truncated GLP-1R. The results show for the first time that seven cysteine

residues in GLP-1R and more than half of the C-terminal tail are not required for GLP-1 binding or function.

2. Material and methods

Receptor constructs - cDNA encoding human GLP-1R was originally obtained from Dr. B. Thorens [11] and subcloned into the mammalian expression vector pcDNA3.1/v5-His-TOPO® (Invitrogen). The presence of the C-terminal His_6-tag was previously shown not to influence the functional response of the receptor [13]. Site-directed mutagenesis of GLP-1R was done using QuikChange™ (Stratagene). The cysteine-deprived GLP-1R, Cys1, contained seven Cys-Ala mutations (Cys^{236}-Ala, Cys^{329}-Ala, Cys^{341}-Ala, Cys^{347}-Ala, Cys^{438}-Ala, Cys^{458}-Ala and Cys^{462}-Ala). The cDNA sequence encoding Cys1 was purchased from GenScript Inc. (New Jersey, USA), and subcloned into pcDNA3.1/v5-His-TOPO®. Cys2, Cys3 and Cys4 were prepared from Cys1 by introducing an additional Cys^{174}-Ala, Cys^{403}-Ala or Cys^{174}-Ala:Cys^{403}-Ala double mutation, respectively. Plasmid DNA was generated using the NucleoBond® Xtra Maxi Plus kit (Macherey-Nagel), and the desired mutations were confirmed by sequencing (Eurofins MWG Operon, Germany).

Cell Culture and Protein Expression– HEK293 cells were maintained in Dulbecco's modified Eagles medium (BioWhittaker) supplemented with 10 vol-% fetal bovine serum (FBS) and 1 vol-% penicillin/streptomycin (100 units/ml) in T-175 flasks (Nunc). HEK293 cells were transiently transfected with 21 µg wild-type or mutant GLP-1R DNA using the FuGENE™ transfection reagent (Roche Applied Science). Cells were harvested 24 hours after transfection and used directly in functional experiments or plasma membrane preparations as previously described [13].

Functional Assay- Transiently transfected HEK293 cells expressing wild-type or mutant GLP-1R were harvested and resuspended in assay buffer incl. IBMX (Flashplate®, Perkin Elmer) to a cell density of 2.4 x 10^6 cells/ml. GLP-1(7-37)-acid was diluted in phosphate buffered saline (PBS) supplemented with 0.02 vol-% Tween-20, and the concentration ranged from 10 fM to 1 nM. Cells in assay buffer (50 µl) and GLP-1 (50 µl) were mixed in 96-well FlashPlates® (Perkin Elmer), gently agitated for 5 minutes and incubated for 25 minutes at room temperature. The resulting intracellular level of cAMP was measured according to supplier's manual and analyzed by non-linear regression/sigmoidal dose-response fitting using Prism 5.0® (GraphPad Software, Inc.).

Receptor Binding Assay- Freshly thawed plasma membrane preparations from transiently transfected HEK293 cells expressing wild-type or mutant GLP-1R were pulled through a 25-gauge needle three times and diluted in assay buffer (50 mM HEPES, 5 mM $MgCl_2$, 5 mM EGTA, 0.005 vol-% Tween-20, pH 7.4). GLP-1(7-37)-acid was diluted in assay buffer, and the concentration ranged from 1 pM to 100 nM. ^{125}I-GLP-1 (7-36)-amide (2.2 Ci/µmol) was

dissolved in assay buffer and added at ~ 50,000 cpm per well corresponding to a final concentration of 50 pM. Nonspecific binding was determined using 1 μM unlabeled GLP-1. Membrane-preparation and radioligand were mixed in 96-well 0.65 μm filter plates (Millipore) and incubated with GLP-1 for 2 hours at 30°C. Subsequently, bound and unbound radioligand were separated by vacuum filtration (Millipore vacuum manifold). The filters were washed twice in 100 μl cold assay buffer and left to dry. Data were analyzed by non-linear regression using Prism 5.0® (GraphPad Software, Inc.). Statistical analyses were also performed in Prism 5.0® using the unpaired t-test.

3. Results

3.1 Substitution of single cysteines

The GLP-1R contains eleven cysteine residues in the TM domain and C-terminal tail. We substituted ten of these cysteines with alanine (Cys^{174} was changed to both Ala and Ser) in order to determine their functional redundancy in GLP-1R. The mutant GLP-1Rs were characterized by their ability to stimulate cAMP production in response to GLP-1 (Table 1). We found that six of the ten Cys-Ala substitutions had no significant negative effect on the GLP-1-mediated response. Several substitutions even increased GLP-1 efficacy significantly and hence, Cys^{236}, Cys^{329}, Cys^{341}, Cys^{347}, Cys^{438}, Cys^{458} and Cys^{462} appeared to be functionally redundant in GLP-1R. However, four Cys-Ala substitutions (Cys^{174}-Ala, Cys^{226}-Ala, Cys^{296}-Ala and Cys^{403}-Ala) significantly reduced GLP-1 potency (Table 1 and Figure 2). Substitution of Cys^{174} with Ala significantly reduced the potency of GLP-1 from 11 ± 3.2 pM to 66 ± 9.5 pM ($p=0.005$) (Figure 2A). Cys^{174} was subsequently substituted with serine, which is more similar to cysteine. However, the Cys^{174}-Ser substitution significantly reduced the potency of GLP-1 from 11 ± 3.2 pM to 79 ± 7.4 pM ($p=0.001$). The Cys^{403}-Ala mutation in TM7 significantly reduced GLP-1 potency from 11 ± 3.2 pM to 59 ± 7.4 pM ($p=0.004$) (Table 1 and Figure 2D).

It was recently shown that Cys^{226} and Cys^{296} are functionally linked through what appears to be a disulfide bond [38]. In the present study, Cys^{226}-Ala showed markedly reduced GLP-1 potency (> 1000 fold compared to wild-type GLP-1R, Table 1 and Figure 2B). E_{max} for GLP-1 was not achieved within the concentration range due to low potency. The Cys^{296}-Ala substitution reduced GLP-1 potency noticeably less compared to Cys^{226}-Ala (Figure 2C). Substituting Cys^{296} with Ala reduced GLP-1 potency 10-fold compared to wild-type GLP-1R ($p=0.02$) but significantly increased GLP-1 efficacy ($p=0.005$) (Table 1). In conclusion, Cys^{236}, Cys^{329}, Cys^{341}, Cys^{347}, Cys^{438}, Cys^{458} and Cys^{462} appear to be functionally redundant in GLP-1R, whereas Cys^{174}, Cys^{226}, Cys^{296} and Cys^{403} seem to be required for the GLP-1-mediated response.

3.2 Development of a fully functional cysteine-deprived GLP-1R

Based on the results of the single Cys-Ala substitutions, we constructed a cysteine-deprived GLP-1R called Cys1. Cys1 contained seven Cys-Ala substitutions (Cys^{236}-Ala, Cys^{329}-Ala, Cys^{341}-Ala, Cys^{347}-Ala, Cys^{438}-Ala, Cys^{458}-Ala and Cys^{462}-Ala) (Figure 3A). Cys2, Cys3 and Cys4 were prepared from Cys1 by introducing an additional Cys^{174}-Ala, Cys^{403}-Ala or Cys^{174}-Ala:Cys^{403}-Ala double mutation, respectively (Figure 3A). The functional properties of the four cysteine-deprived GLP-1Rs are shown in Figure 3B.

The potency and affinity of GLP-1 was similar for Cys1 compared to wild-type GLP-1R (EC_{50}= 11 ± 2.3 pM and IC_{50}= 0.3 ± 0.02 nM for Cys1 compared to EC_{50}= 9 ± 2.2 pM and IC_{50}= 1.0 ± 0.2 nM for wild-type GLP-1R). However, the expression level of Cys1 appeared to be reduced to ~ 60% of wild-type GLP-1R in transiently transfected HEK293 cells based on specific binding of ^{125}I-GLP-1 (data not shown). Cys2 contained an additional Cys^{174}-Ala substitution in ICL1, and Cys4 contained an additional Cys^{174}-Ala:Cys^{403}-Ala double mutation compared to Cys1. This completely abolished GLP-1-mediated cAMP production (Figure 3B). Cys3 contained an additional Cys^{403}-Ala substitution in TM7 compared to Cys1, which reduced to potency of GLP-1 noticeably (from EC_{50}= 9 ± 2.2 pM on wild-type GLP-1R to EC_{50}= 242 ± 81 pM on Cys3). Hence, seven cysteine residues (Cys^{236}, Cys^{329}, Cys^{341}, Cys^{347}, Cys^{438}, Cys^{458} and Cys^{462}) were dispensable in GLP-1R, and simultaneous substitution of these cysteines with alanine had no effect on GLP-1 binding or function.

3.3 Truncation of the GLP-1R C-terminal

We prepared a series of extensive deletions in the C-terminal tail of wild-type GLP-1R in order to determine the limit for truncation (Figure 4). The truncated receptors were initially characterized by their ability to stimulate cAMP production in response to GLP-1, and fully functional receptors were subsequently characterized by their ability to bind GLP-1 (Table 2 and Figure 5). In order to confirm the results of a previous GLP-1R truncation study [34], we deleted the last 44 amino acids of wild-type GLP-1R. However, the 1-419 truncation mutant significantly reduced the potency of GLP-1 from EC_{50}= 17 ±3.3 pM for wild-type GLP-1 to EC_{50}= 106 ± 13 pM for 1-419 ($p = 0.02$) (Figure 5A). More extensive deletions completely abolished GLP-1-mediated cAMP production (Table 2). Conversely, the reduced potency of GLP-1 for the 1-419 truncation mutant was almost completely restored by the presence of Trp^{420}, as the GLP-1-mediated responses of 1-420 and 1-421 were comparable to wild-type GLP-1R (Table 2). However, the specific binding of ^{125}I-GLP-1 was dramatically reduced for the 1-421 truncation mutant (~ 6% compared to wild-type GLP-1R, Figure 6), which suggested low surface expression of the receptor. The presence of Leu^{422} increased the specific binding to 32%, and the presence of Leu^{422}-His^{426} increased the specific binding of ^{125}I-GLP-1 to 65% compared to wild-type GLP-1R (Figure 6). Despite reduced binding of ^{125}I-GLP-1, the 1-422

and 1-426 truncation mutants displayed similar potency and affinity for GLP-1 compared to wild-type GLP-1R (Table 2 and Figure 5). Hence, Leu^{422} appears to mark the limit for C-terminal truncation of wild-type GLP-1R, although this probably compromises receptor expression. Cys1 was subsequently truncated after Leu^{422} and His^{426} respectively, and the truncated receptors were characterized by their ability to bind GLP-1 and stimulate cAMP production in response to GLP-1 (Table 2 and Figure 5). The specific binding of ^{125}I-GLP-1 to $Cys1_{1\text{-}422}$ and $Cys1_{1\text{-}426}$ was reduced to a level comparable to the specific binding of ^{125}I-GLP-1 to 1-422 and 1-426 (Figure 6). This reduced the potency and efficacy of GLP-1 for the $Cys1_{1\text{-}422}$ mutant although the reduction did not reach statistical significance. The affinity of GLP-1 for the $Cys1_{1\text{-}422}$ mutant could not be determined due to poor definition of the top-and bottom plateau of the binding curve, which may in turn be a result of reduced surface expression of the receptor. In contrast, the potency and affinity of GLP-1 for the $Cys1_{1\text{-}426}$ mutant was similar to wild-type GLP-1R (Table 2).

4. Discussion

4.1 The importance of cysteines in the TM domain of GLP-1R

The aim of our work was to develop a fully functional cysteine-deprived and C-terminally truncated GLP-1R. GLP-1R contains seventeen cysteine residues (Figure 1) of which six are essential for maintaining the tertiary structure of ECD [17]. The remaining eleven cysteines are found in the transmembrane (TM) helices, the loops and in the intracellular C-terminal tail. According to the existing literature, only Cys^{329} has been shown to be redundant [26]. We substituted the remaining ten cysteines with alanine (Cys^{174} was changed to both Ala and Ser) in order to determine their functional redundancy in GLP-1R. Based on the substitution of single cysteines, we found that six of the ten Cys-Ala substitutions had no significant negative effect on the GLP-1-mediated response and hence, Cys^{236}, Cys^{329}, Cys^{341}, Cys^{347}, Cys^{438}, Cys^{458} and Cys^{462} appeared to be functionally redundant in GLP-1R. The reduced efficacy of GLP-1 reported previously for the Cys^{438}-Ala substitution [27] was not observed in this study.

The remaining four cysteines (Cys^{174}, Cys^{226}, Cys^{296} and Cys^{403}) significantly reduced GLP-1 potency when changed to alanine. Cys^{174} resides in ICL1, distant from the putative binding site of GLP-1. Nevertheless, substitution of Cys^{174} with Ala significantly reduced the potency of GLP-1. This could indicate that Cys^{174} helps to stabilize an active conformation of the receptor. However, Mathi *et al.* previously showed that the Cys^{174}-Ala mutation significantly reduced the expression level of GLP-1R [26], which correlates well with the reduced potency of GLP-1 observed in this study. The increased efficacy of GLP-1 cannot immediately be explained based on these results. Cys^{174} was subsequently changed to serine, which is more similar to cysteine. However, this substitution significantly reduced the potency of GLP-1, demonstrating the importance of a cysteine in ICL1. The presence of a cysteine in TM7 may also be important for

GLP-1 function and/or expression of GLP-1R, as the Cys^{403}-Ala mutation significantly reduced GLP-1 potency.

It was recently shown that Cys^{226} and Cys^{296} are functionally linked through what appears to be a disulfide bond [38] similar to the disulfide bond connecting ECL1/TM3 and ECL2 in family A [21-23] and other family B GPCRs [24;25]. Our results confirm that Cys^{226} is important for the structural integrity of GLP-1R TM domain and/or GLP-1 binding and function. Interestingly, the Cys^{296}-Ala substitution reduced GLP-1 potency noticeably less compared to Cys^{226}-Ala. Hence, lack of functional coupling between ECL1 and ECL2 appears to have a more pronounced effect on GLP-1 function if Cys^{226} is replaced by Ala compared to Cys^{296}. A similar observation was made recently for the rat GLP-1R [38], but the loss-of-function effect of the Cys^{226}-Ala mutation was restored by additional substitution of Cys^{296} to Ala. The loss of GLP-1 potency observed for the Cys^{226}-Ala mutant was thus explained as a result of the released, bulky Cys^{296} rather than a result of an absent disulfide bond [38]. It may be that GLP-1 function is affected more by the putative release of Cys^{296} compared to the release of Cys^{226} because Cys^{296} resides closely to the binding/activation pocket of GLP-1. In this way, the presence of a free, bulky cysteine in position 296 (in the Cys^{226}-Ala mutant) would have a more significant effect on GLP-1 potency compared to a free Ala (in the Cys^{296}-Ala mutant). The presence of a free Ala in position 296 would reduce GLP-1 function compared to wild-type GLP-1R where rotation of Cys^{296} is restricted by a disulfide bond to Cys^{226}. This restriction may keep Cys^{296} away from the GLP-1 binding/activation pocket in the wild-type receptor, and would explain why the Cys^{296}-Ala mutation reduces GLP-1 potency by 10-fold. However, a recent publication enhances the significance of Cys^{296} by showing that a Cys^{296}-Ala substitution decreases GLP-1 potency for the cAMP pathway by 100-fold and at the same time enhances GLP-1 signaling bias for phosphorylation of ERK1/2 [39]. In conclusion, the single cysteine substitutions show that Cys^{236}, Cys^{329}, Cys^{341}, Cys^{347}, Cys^{438}, Cys^{458} and Cys^{462} appear to be functionally redundant in GLP-1R, whereas Cys^{174}, Cys^{226}, Cys^{296} and Cys^{403} may be required for the GLP-1-mediated response.

In order to investigate whether the seven redundant cysteine residues could be removed from GLP-1R simultaneously and whether this affected the function of Cys^{174} and Cys^{403}, we constructed a cysteine-deprived GLP-1R called Cys1 (Figure 3A). Cys2, Cys3 and Cys4 were prepared from Cys1 by introducing an additional Cys^{174}-Ala, Cys^{403}-Ala or Cys^{174}-Ala:Cys^{403}-Ala double mutation, respectively (Figure 3A). The potency and affinity of GLP-1 was similar for Cys1 compared to wild-type GLP-1R, which confirmed the functional redundancy of the seven cysteine residues. However, changing a single cysteine in Cys1 to alanine (Cys^{174}), completely abolished GLP-1-induced cAMP production (Cys2). Simultaneous substitution of Cys^{403} with alanine (Cys4) did not change to functionality of the receptor, which confirmed the importance of Cys^{174} for the structural integrity and/or activation of GLP-1R. The reduced GLP-1 potency of

Cys3 established that Cys^{403} may also be important for GLP-1 function and/or expression of GLP-1R.

4.2 A fully functional cysteine-deprived and C-terminally truncated GLP-1R

FRET based studies have shown that the intracellular C-terminal part of β_2AR is flexible and unstructured [31], and several family A GPCRs have been C-terminally truncated to facilitate crystallization [28-30]. The distal part of GLP-1R C-terminus appears to be important for desensitisation and internalisation of the receptor [37], but not for G-protein-mediated signaling [34]. It was previously reported that GLP-1R can be truncated after Arg^{419} without affecting GLP-1 affinity or potency [34]. This study served as a starting point for the C-terminal truncations described in this paper. However, we were not able to reproduce the findings of Vázquez *et al.*, as the 1-419 truncation mutant significantly reduced GLP-1 potency. More extensive deletions completely abolished GLP-1-mediated cAMP production, suggesting that the membrane proximal part of GLP-1R C-terminal is important for expression of GLP-1R at the cell surface and/or receptor activation. Interestingly, biophysical studies of a peptide mimic of the membrane proximal part of the calcitonin receptor-like receptor (CLR) C-terminal indicated that this region forms a membrane-anchored α-helix similar to helix 8 of family A GPCR [40]. The putative helix 8 of CLR was subsequently found to be involved in G_s-coupling and cell surface expression of the receptor [41]. The latter has also been shown for the membrane proximal part of GLP-2R C-terminal [35]. It must be noted, however, that the C-terminal of CRFR1 is completely dispensable [42], so an eighth helix may not be present in all family B GPCRs. In line with the role of the membrane proximal part of the C-terminal described for other family B GPCRs, we observed a direct correlation between the length of the C-terminal and the specific binding of ^{125}I-GLP-1. Accordingly, our results support the notion that the membrane proximal part of the C-terminal tail could be involved in transport of GLP-1R to the cell surface. The reduced binding of ^{125}I-GLP-1 did not affect the affinity or potency of GLP-1 for the 1-422 and 1-426 truncation mutants. Hence, Leu^{422} appears to mark the limit for C-terminal truncation of wild-type GLP-1R, although this probably compromises receptor expression.

Based on the results obtained with C-terminal truncation of the wild-type GLP-1R, Cys1 was truncated after Leu^{422} and His^{426}, respectively. As for the wild-type receptor, we observed a direct correlation between the length of the C-terminal and the specific binding of ^{125}I-GLP-1. Interestingly, the specific binding of ^{125}I-GLP-1 was reduced to a level comparable to 1-422 and 1-426 in wild-type GLP-1R despite reduced specific binding/expression of the template receptor, Cys1 (data not shown). Hence, the specific binding of ^{125}I-GLP-1 was comparable for Cys1 and for the truncated $Cys1_{1\text{-}426}$ mutant, indicating that Cys1 is less sensitive to C-terminal truncation with regards to cell surface expression. Conversely, Cys1 seems more sensitive to

C-terminal truncation with regards to receptor functionality, than the wild-type GLP-1R. The potency of GLP-1 was reduced for the $Cys1_{1-422}$ mutant although the specific binding of ^{125}I-GLP-1 was comparable to that of the 1-422 wild-type mutant. In contrast, the potency and affinity of GLP-1 for the $Cys1_{1-426}$ mutant was similar to wild-type GLP-1R, which established that seven cysteines and the last 37 residues of the C-terminal tail can be removed from GLP-1R without compromising GLP-1 binding or function.

Conclusion- The present study describes the development of a cysteine-deprived and C-terminally truncated GLP-1R that retains full GLP-1 potency and affinity ($Cys1_{1-426}$). The results show that seven cysteines and more than half of the C-terminal tail are not required for GLP-1 binding or function. Presence of an eighth helix in GLP-1R has not yet been reported, but the results presented here indicate that the membrane proximal portion of the C-terminal tail is important for expression of GLP-1R at the cell surface.

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Contributions

C.R.U. undertook the experimental work and wrote the manuscript. L.B.K., P.W.G., G.H.P. and S. R-R. contributed to the discussion.

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Legends

Table 1. Functional properties of wild-type and cysteine-substituted GLP-1Rs

All values represent mean ± SEM of three independent experiments performed in duplicates. The EC_{50} values of GLP-1 are given in pM, and GLP-1 efficacy (E_{max}) is given in pmol cAMP. The EC_{50} and E_{max} values of GLP-1 on mutant receptors were compared to those of wild-type GLP-1R individually using the unpaired t-test. * indicates $p < 0.05$ and ** indicates $p < 0.01$. (a) The E_{max} value was calculated by Prism 5.0® (GraphPad Software, Inc.) even though the top plateau of the concentration-response curve was not well-defined. The top of the curve was fitted by obtaining the best symmetry with the lowest sum-of-squares. ND: Not detectable within the concentration range due to low potency.

Table 2. Functional properties of wild-type and C-terminally truncated GLP-1Rs

All values represent mean ± SEM of 2-6 independent experiments performed in duplicates. Agonist ^{125}I-GLP-1 tracer was used for competition binding experiments. The EC_{50}, E_{max} and IC_{50} values of GLP-1 for the mutant receptors were compared to those of wild-type GLP-1R individually using the unpaired t-test. * indicates $p = 0.02$. (a) The E_{max} value was calculated by Prism 5.0® (GraphPad Software, Inc.) even though the top plateau of the concentration-response curve was not well-defined. The top of the curve was fitted by obtaining the best symmetry with the lowest sum-of-squares. ND: Not detectable, possibly due to low surface expression.

Figure 1. Cysteine residues in GLP-1R

Snake diagram of GLP-1R showing the position and numbering of the seventeen cysteine residues (white letters in black circles) in the receptor. The putative disulfide bond between Cys^{226} and Cys^{296} is indicated with a dashed line. The predicted signal sequence (light grey) [43] and putative glycosylation sites (Y) [11] are also shown.

Figure 2. Concentration-response curves for cysteine-substituted GLP-1Rs with reduced GLP-1 potency

GLP-1-induced cAMP production in HEK293 cells expressing the Cys^{174}-Ala (A), Cys^{226}-Ala (B), Cys^{296}-Ala (C) or Cys^{403}-Ala (D) mutant GLP-1Rs. Red curves show the GLP-1-induced response of mutant receptors compared to that of the wild-type GLP-1R (black dashed curve). Data represent the average of three independent experiments for each receptor mutant.

Figure 3. Amino acid sequence and functional properties of four cysteine-deprived GLP-1Rs

(A) Amino acid sequence of Cys1-Cys4. Cys1 contained seven Cys-Ala substitutions (white letters in black circles). Cys2, Cys3 and Cys4 were prepared from Cys1 by introducing an additional Cys^{174}-Ala, Cys^{403}-Ala or Cys^{174}-Ala:Cys^{403}-Ala double mutation, respectively (black letters in gray circles). (B) Stimulation of cAMP production by GLP-1 in HEK293 cells transiently expressing wild-type or Cys1-Cys4 GLP-1Rs. Data represent one of two independent experiments performed in duplicate. (C) ^{125}I-GLP-1 displacement by GLP-1 on plasma membranes from HEK293 cells transiently expressing wild-type (black dashed curve) or Cys1 GLP-1R (red curve). Data are normalized according to ^{125}I-GLP-1 binding and represent one of three independent experiments performed in duplicate.

Figure 4. Snake diagram of GLP-1R C-terminal tail

Seven extensive deletions were made in the wild-type GLP-1R C-terminal tail. Arrowheads indicate deletion sites.

Figure 5. Functional- and binding properties of C-terminally truncated GLP-1Rs

(A) Stimulation of cAMP production by GLP-1 in HEK293 cells transiently expressing wild-type or truncated GLP-1Rs. Data represent 3-5 independent experiments performed in duplicate. (B) ^{125}I-GLP-1 displacement by GLP-1 on plasma membranes from HEK293 cells transiently expressing wild-type or truncated GLP-1R. Data are normalized according to ^{125}I-GLP-1 binding and represent 2-6 independent experiments performed in duplicate.

Figure 6. Specific binding of ^{125}I-GLP-1 to C-terminally truncated GLP-1Rs

Plasma membranes from transiently transfected HEK293 cells expressing wild-type GLP-1R or truncated GLP-1Rs were incubated with 1 pM GLP-1 and 50 pM ^{125}I-GLP-1 for 2 hours at 30°C. Nonspecific binding was determined using 1 μM GLP-1. Specific binding of ^{125}I-GLP-1 to each of the truncated GLP-1Rs was compared to that of wild-type GLP-1R using the unpaired t-test. *** indicates $p<0.0001$.

Table 1

Receptor	GLP-1 EC_{50} (pM)	$EC_{50(wt)}$/ $EC_{50(mutant)}$	GLP-1 E_{max} (pmol cAMP)
Wild-type	*11 ± 3.2*	*1*	*13 ± 2.3*
Cys^{174}-Ala (ICL1)	66 ± 9.5**	0.17	23 ± 2.6*[a]
Cys^{174}-Ser (ICL1)	79 ± 7.4**	0.14	19 ± 3.0
Cys^{226}-Ala (ECL1)	> 1000	> 0.01	ND
Cys^{236}-Ala (TM3)	28 ± 10	0.39	30 ± 2.6**
Cys^{296}-Ala (ECL2)	115 ± 27*	0.10	32 ± 2.1**[a]
Cys^{341}-Ala (ICL3)	14 ± 2.6	0.79	22 ± 1.7*
Cys^{347}-Ala (ICL3/TM6)	10 ± 1.9	1.1	22 ± 1.8*
Cys^{403}-Ala (TM7)	59 ± 7.2**	0.19	20 ± 4.5
Cys^{438}-Ala (C-terminal)	27 ± 8.2	0.41	17 ± 4.7
Cys^{458}-Ala (C-terminal)	28 ± 6.3	0.39	16 ± 3.8
Cys^{462}-Ala (C-terminal)	28 ± 7.1	0.39	19 ± 0.88

Table 2

Receptor	GLP-1 EC_{50} (pM)	GLP-1 E_{max} (pmol cAMP)	GLP-1 IC_{50} (nM)
Wild-type	17 ± 3.3	21 ± 4.4	0.6 ± 0.2
1-410	NA	NA	-
1-414	NA	NA	-
1-419	106 ± 13*	21 ± 3.7[a]	-
1-420	35 ± 8.9	28 ± 1.5	-
1-421	42 ± 15	31 ± 6.0	ND
1-422	13 ± 2.0	22 ± 2.6	0.5 ± 0.2
1-426	10 ± 3.0	17 ± 2.0	0.6 ± 0.2
$Cys1_{1-422}$	58 ± 31	14 ± 1.9	ND
$Cys1_{1-426}$	23 ± 9.0	26 ± 2.9	0.4 ± 0.1

Figure 1

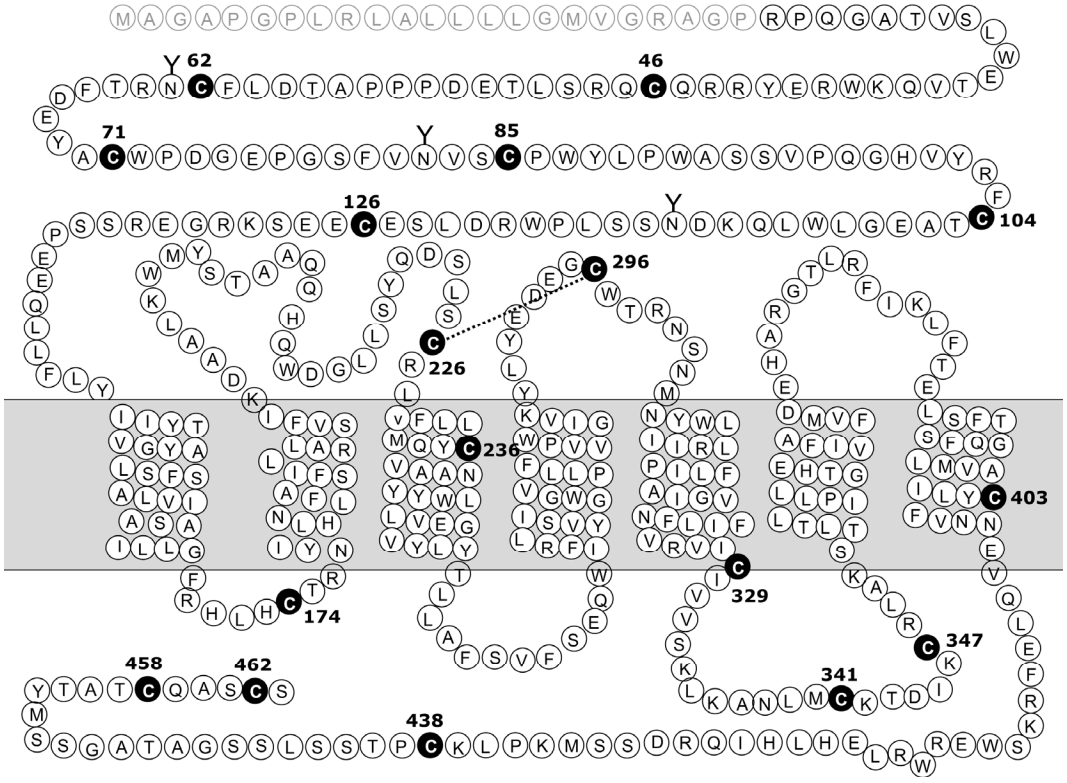


Figure 2

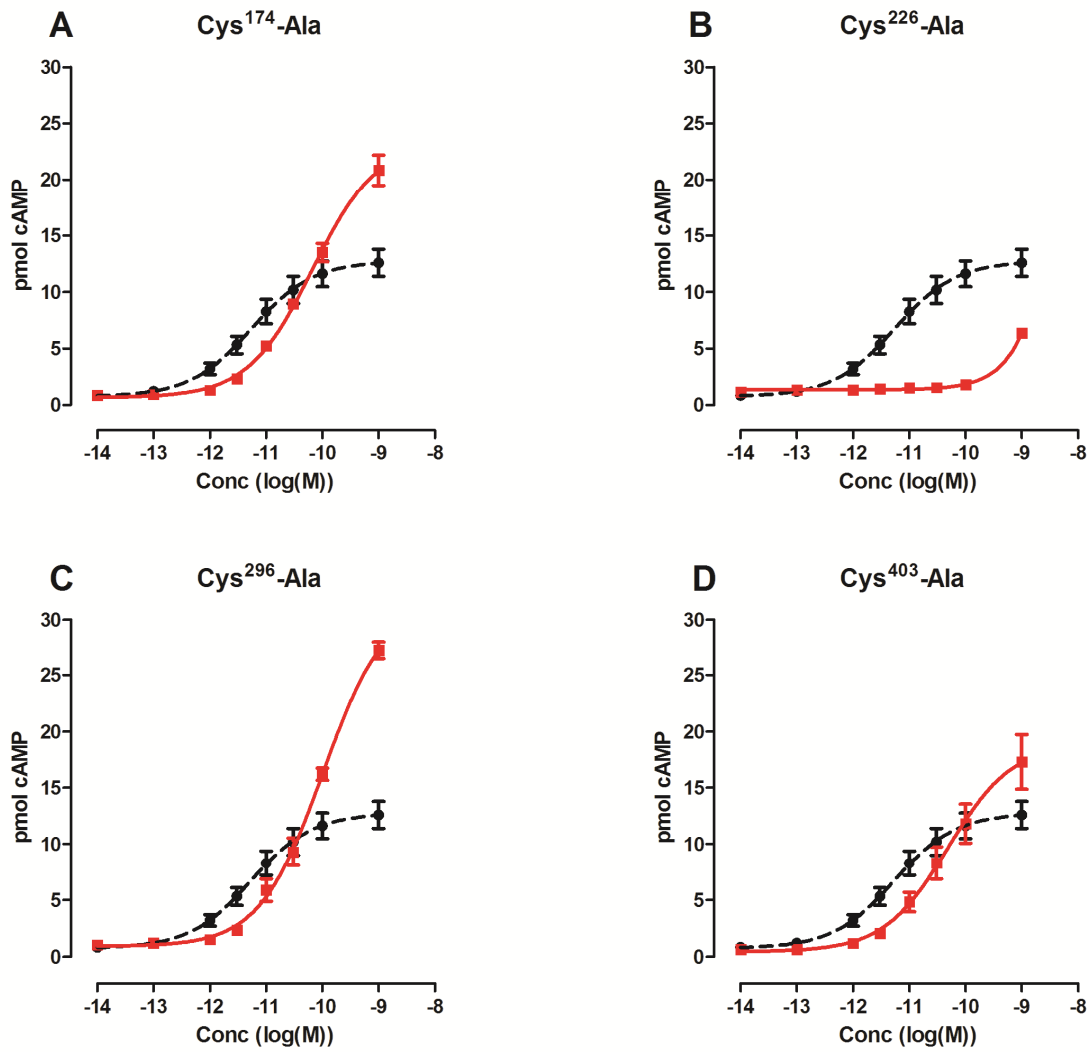

A
Cys174-Ala
B
Cys226-Ala
C
Cys296-Ala
D
Cys403-Ala
pmol cAMP
Conc (log(M))
30
25
20
15
10
5
0
-14
-13
-12
-11
-10
-9
-8

Figure 3

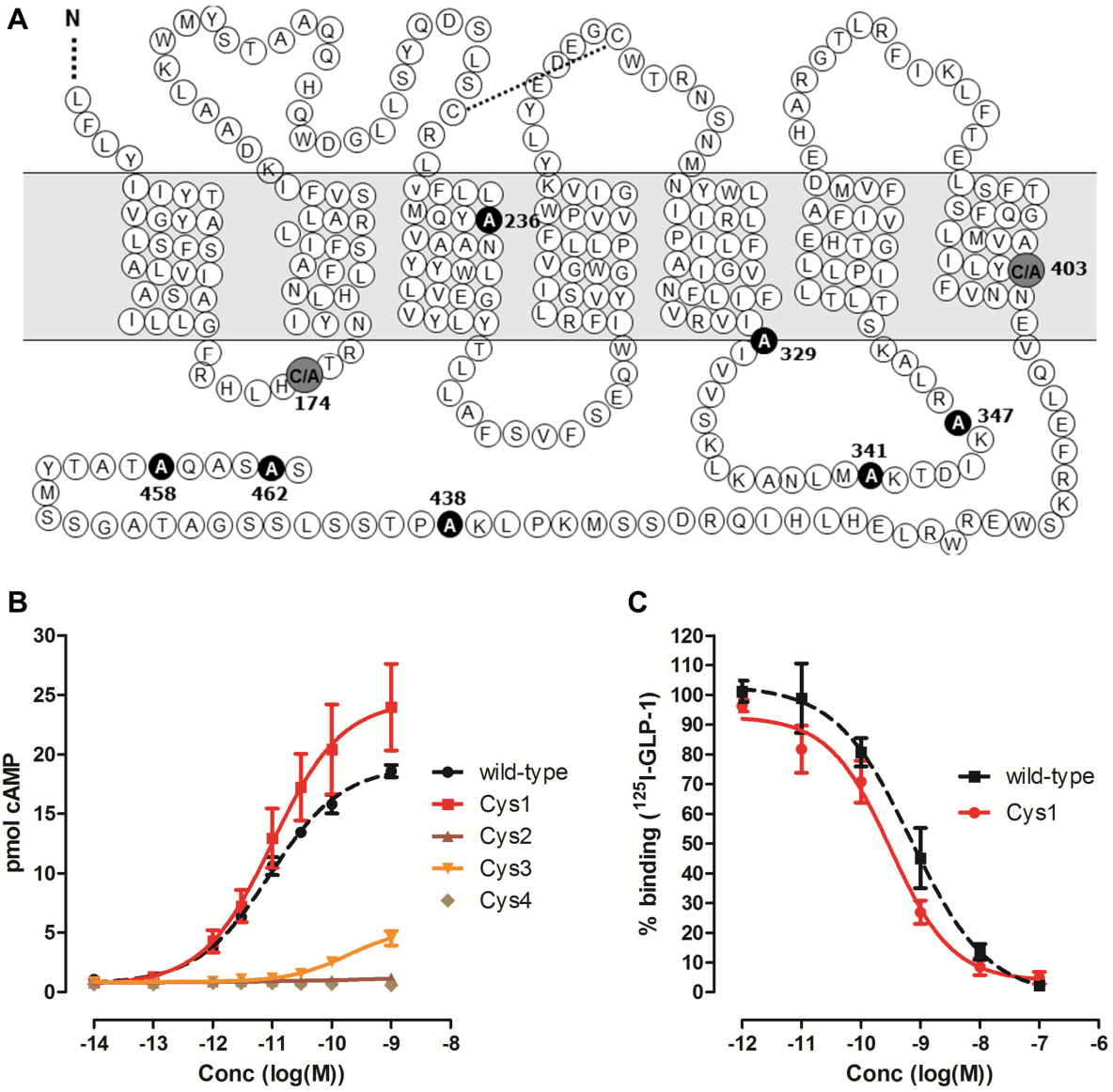

A
N
C/A
174
C/A
403
236
329
347
341
458
462
438
B
pmol cAMP
wild-type
Cys1
Cys2
Cys3
Cys4
Conc (log(M))
C
% binding (^{125}I-GLP-1)
wild-type
Cys1
Conc (log(M))

Figure 4

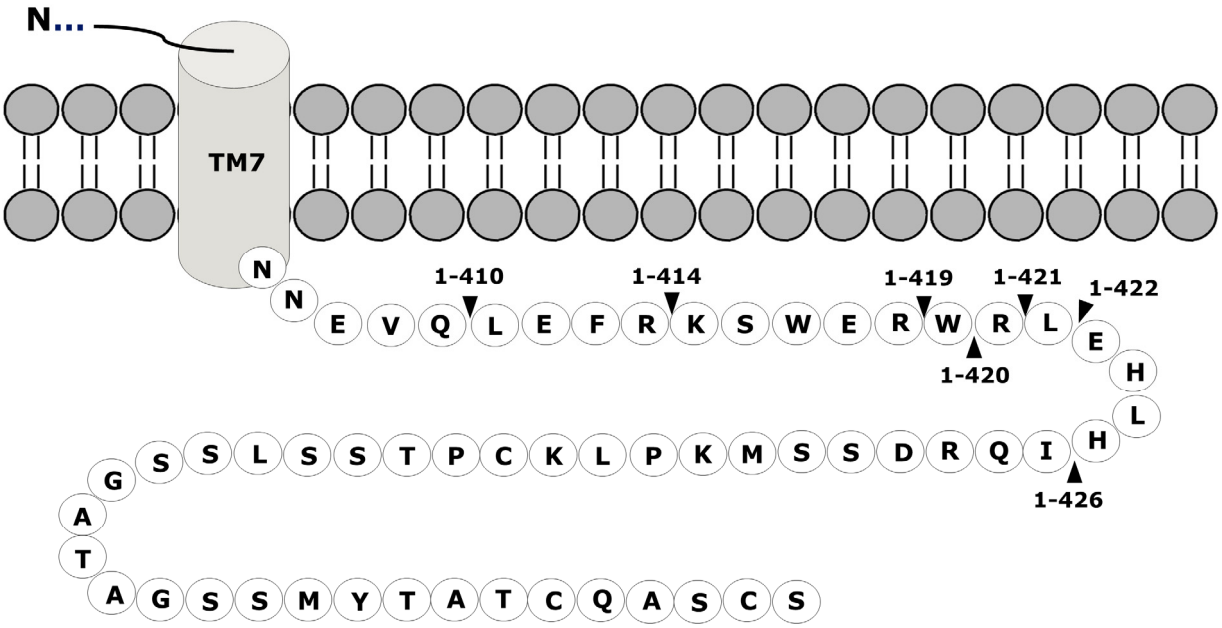

N...
TM7
N
N
E V Q L E F R K S W E R W R L E H L
1-410
1-414
1-419
1-421
1-422
1-420
1-426
S S L S S T P C K L P K M S S D R Q I H
G S
A
T
A G S S M Y T A T C Q A S C S

Figure 5

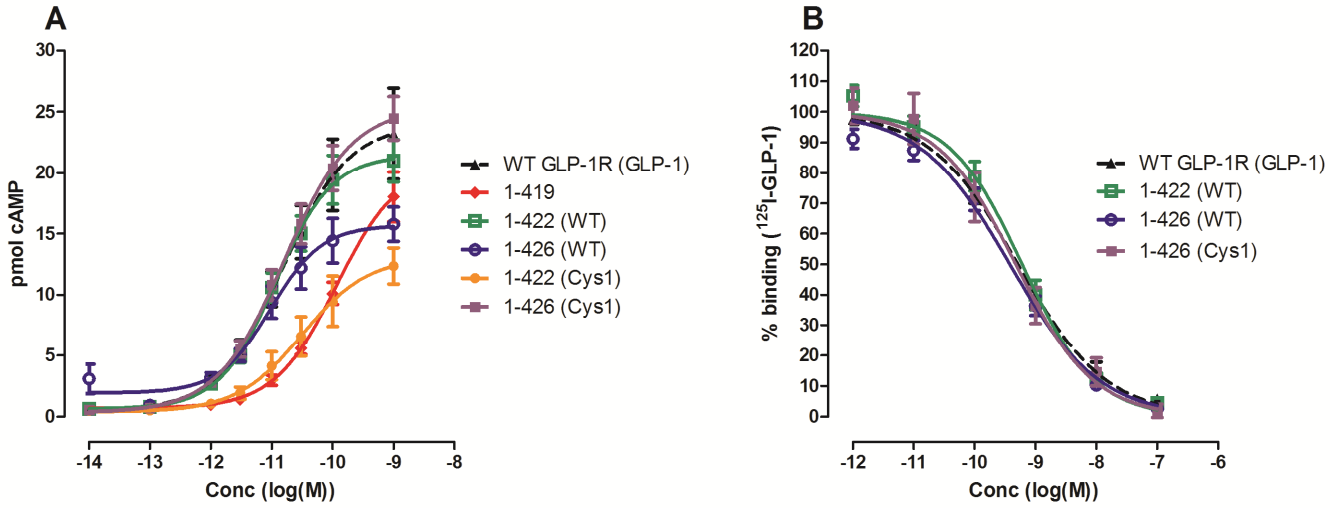
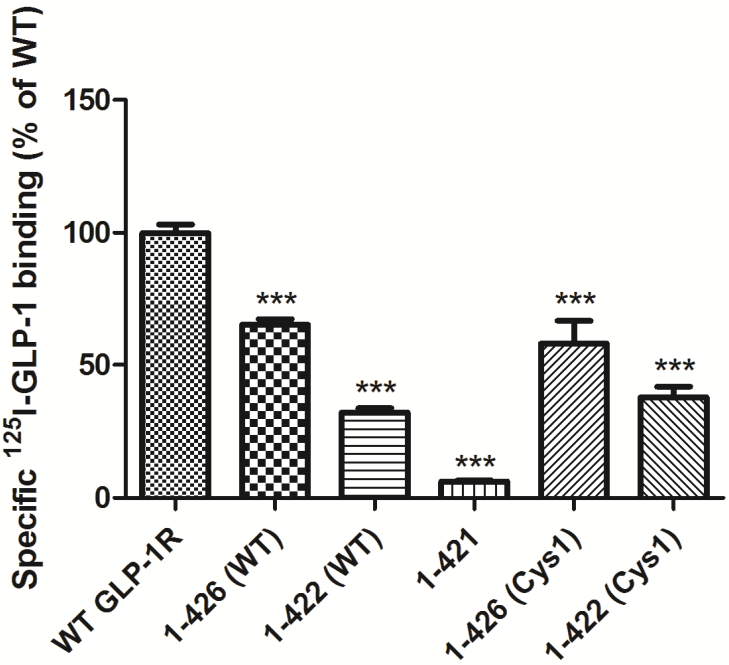

A
pmol cAMP
30
25
20
15
10
5
0
-14
-13
-12
-11
-10
-9
-8
Conc (log(M))
WT GLP-1R (GLP-1)
1-419
1-422 (WT)
1-426 (WT)
1-422 (Cys1)
1-426 (Cys1)
B
% binding (^{125}I-GLP-1)
120
110
100
90
80
70
60
50
40
30
20
10
0
-12
-11
-10
-9
-8
-7
-6
Conc (log(M))
WT GLP-1R (GLP-1)
1-422 (WT)
1-426 (WT)
1-426 (Cys1)

Figure 6


Specific 125I-GLP-1 binding (% of WT)
150
100
50
0
***
***
***
***
***
WT GLP-1R
1-426 (WT)
1-422 (WT)
1-421
1-426 (Cys1)
1-422 (Cys1)

Study IV – Real-time cAMP profiles of GLP-1R agonists

A part of the Ph.D. studies included secondment to the lab of Prof. Hans Bräuner-Osborne, Dept. of Drug Design and Pharmacology, University of Copenhagen. The group is currently optimising the use of Epac-based cAMP biosensors as a tool to study GPCR pharmacology. The purpose of the Ph.D. work was to study the cAMP kinetics of GLP-1R upon stimulation with different receptor agonists. The results suggested that exendin-4 has a prolonged effect on GLP-1R compared to other ligands. Subsequent experiments indicated that this could be a result of superior enzymatic stability of exendin-4, but further investigations are required to confirm this. Some of the results obtained in Study IV are included in a co-authored paper that is currently in preparation (226). I undertook all the experimental work included in Study IV apart from the Luminescent Oxygen Channeling Immunoassay (LOCI), which was performed by Christian Rosenquist, Research Bioanalysis, Novo Nordisk A/S.

Introduction to cAMP biosensors

GLP-1R preferably couples to the G_s-protein (194) which leads to activation of AC and consequently to the formation of cAMP from ATP. Quantification of intracellular cAMP levels is a central technique in pharmacological characterisation of GPCRs, and numerous methods have been developed over the years. The functional receptor assay used to quantify cAMP levels in Study I, Study II and Study III (FlashPlate®, Perkin Elmer) is an immunobased assay, where a known amount of labelled cAMP compete with non-labelled cell-derived cAMP for a fixed number of binding sites (anti-cAMP antibody) (supplier's manual). Like many other commercially available functional assays, the FlashPlate® assay is used to quantify the accumulated amount of intracellular cAMP over a fixed period (endpoint cAMP). However, over the past years, different FRET-based cAMP biosensors have been developed (243-245). The biosensors enable continuous monitoring of cAMP as it is produced and degraded (real-time cAMP), which offers a more accurate and sensitive approach to study GPCR activation. In addition, a biosensor can be tagged with a signal sequence that localises the sensor to a specific subcellular compartment, which in turn facilitates spatial and temporal monitoring of cAMP (244). The first cAMP biosensors used a fluorescent probe based on Protein kinase A (PKA) (245). However, in recent years, fluorescently labelled 'Exchange protein directly activated by cAMP' (Epac) has become widely used as a cAMP biosensor (245). Epac1 and Epac2 are two closely related guanine nucleotide exchange factors (GEFs) for the small GTPases Rap1 and Rap2. Epac is activated directly by cAMP, and Epac1 has one binding site for cAMP, whereas Epac2 has two (246;247). The first generation of Epac-based cAMP biosensors used full-length Epac1 fused to enhanced cyan fluorescent protein (ECFP) in the N-terminal and Citrine in the C-terminal (244). Upon binding of cAMP, Epac changes conformation, which increases the distance between the fluorophores and consequently changes the FRET ratio. The Epac biosensor has subsequently been optimised to obtain a more even distribution of fluorescence in the cytoplasm. Native Epac is mainly localised in the nuclear membrane and mitochondria (248), which resulted in different subcellular distributions of the fluorescent signal using full-length Epac (244). Deletion of the first N-terminal 148 amino acids of Epac1 yielded a cytosolic protein (248), which retained full cAMP binding affinity, and thus was more suitable as a cAMP biosensor than full-length Epac (249). The fluorophores have also been optimised since the first Epac-based biosensors were constructed (250), and more efficient fluorescent FRET pairs are now used to flank the cAMP binding domain of Epac1 (251).

Prof. Hans Bräuner-Osborne's group has constructed a cAMP biosensor consisting of Epac1 (amino acids 149-881) fused N-terminally to the monomeric form of Cerulean (mCer) and C-terminally to the monomeric form of Citrine (mCit). A detailed description of the biosensor construct (Epac149, Figure 16) and its characterisation can be found in (251). Cerulean is a brighter variant of ECFP, which has been optimised specifically for use in FRET experiments (250), whereas Citrine is a modified form of yellow fluorescent protein (YFP) with increased brightness and photostability (252).

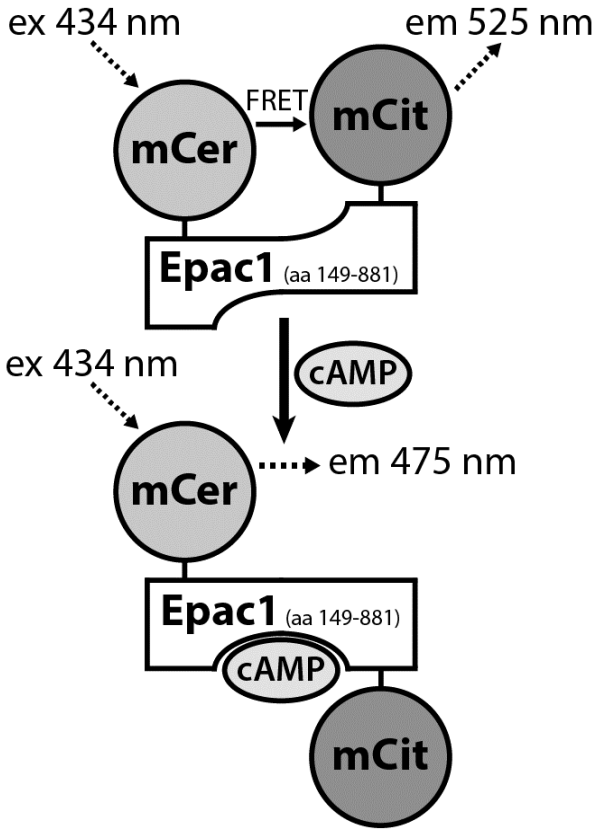


Figure 16. The Epac149 biosensor. Upon binding of cAMP to the Epac149 biosensor, the distance between the two fluorophores mCer and mCit increases, which changes the FRET signal i. e. the em474/em524 ratio is increased. The figure was kindly provided by Jesper M. Mathiesen, University of Copenhagen.

Experimental Procedures

Cell Culture and Protein Expression – HEK293 cells were maintained in Dulbecco's modified Eagles medium (BioWhittaker) supplemented with 10 vol-% fetal bovine serum (FBS) and 1 vol-% penicillin/streptomycin (100 units/ml) in 15 cm dishes. HEK293 cells were transiently co-transfected with 0.1, 0.5, 10 or 21 µg GLP-1R and 21 µg Epac149 DNA using the FuGENE^{TM} transfection reagent (Roche Applied Science). The GLP-1R construct was described previously (175). Cells were harvested 24 hours after transfection and the transfection efficiency was quantified by means of fluorescence microscopy. Cells were used in the real-time cAMP assay after harvesting.

Real-time cAMP assay - Transiently transfected HEK293 cells expressing GLP-1R and Epac149 were harvested and resuspended in assay buffer (Hank's Buffered Salt Solution (HBSS) supplemented with 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.02% Tween-20, pH 7.4) supplemented with 0.3 vol-% bovine serum albumin (BSA) to a cell density of ~ 700.000

fluorescent counts (EnVision Multilabel Reader) for high expression levels. GLP-1(7-37), exendin-4, glucagon, oxyntomodulin, taspoglutide and exendin-4(9-39) were diluted in assay buffer. Cells in assay buffer (20 µl) and ligand (20 µl) (with or without 10 µM valine pyrrolidide (Valpyr)) were mixed in black 384-well plates (Corning) and the resultant fluorescence of mCer and mCit was measured after excitation of mCer in an EnVision Multilabel Reader (Perkin Elmer) at room temperature. For antagonist experiments, a final concentration of 1 µM exendin-4(9-39) was added 5, 30 or 300 minutes after adding the cells to GLP-1. Reading of the plate was initiated as soon as possible after adding the cells in order to obtain the initial kinetics of cAMP production. The FRET ratio (em524 nm/em474 nm) was measured every 10 minutes for at least 300 minutes (5 hours) in the presence of agonist alone or when exendin-4(9-39) was added after 300 minutes. The FRET ratio was measured every 2 minutes for at least 2 hours in all other experiments containing agonist and antagonist. For illustration purposes, the FRET ratio was inverted (em474 nm/em524 nm), and the ratio was plotted as a function of time using Prism 5.0® (GraphPad Software, Inc.).
Apparatus - EnVision Multilabel Reader (Perkin Elmer) equipped with a CFP/YFP dichroic mirror and the following filters (251): Excitation filter for mCer: CFP 430 (barcode 138), emission filter for mCer: CFP 470 (barcode 240) and emission filter for mCit: YFP 535 (barcode 274).
LOCI – Stability of GLP-1 and exendin-4 over time was estimated by adding 1 nM ligand (100 µl) to HEK293 cells transiently expressing GLP-1R and Epac149 in duplicates. Cells were resuspended in assay buffer (100 µl) to a cell density of ~ 700.000 fluorescent counts (EnVision Multilabel Reader). Ligand and cells were incubated for 0, 30, 60, 90, 120, 150, 200, 250 or 300 minutes followed by centrifugation at 10.000 g for 5 minutes. The supernatant was frozen immediately on dry ice. Samples were analysed using LOCI. Donor beads were coated with streptavidin, whereas acceptor beads were conjugated with a monoclonal antibody specific for the C-terminus of the ligand. The second monoclonal antibody, recognizing an N-terminus epitope, was biotinylated. The three reactants were combined with the ligand and formed a two-sited immuno-complex. Illumination of the complex released singlet oxygen atoms from the donor beads, which were channeled into the acceptor beads and triggered a chemiluminescence response, which was measured in an EnVision Multilabel Reader (Perkin Elmer).

Results and Discussion

cAMP profiles of high- and low potency GLP-1R agonists
GLP-1, exendin-4, glucagon and oxyntomodulin were added to HEK293 cells transiently expressing GLP-1R and Epac149, and the resultant FRET ratio (em524 nm/em474 nm) was measured every 10 minutes for 300 minutes (5 hours). For illustration purposes, the FRET ratio was inverted, and the cAMP profiles of the four agonists are shown in Figure 17.

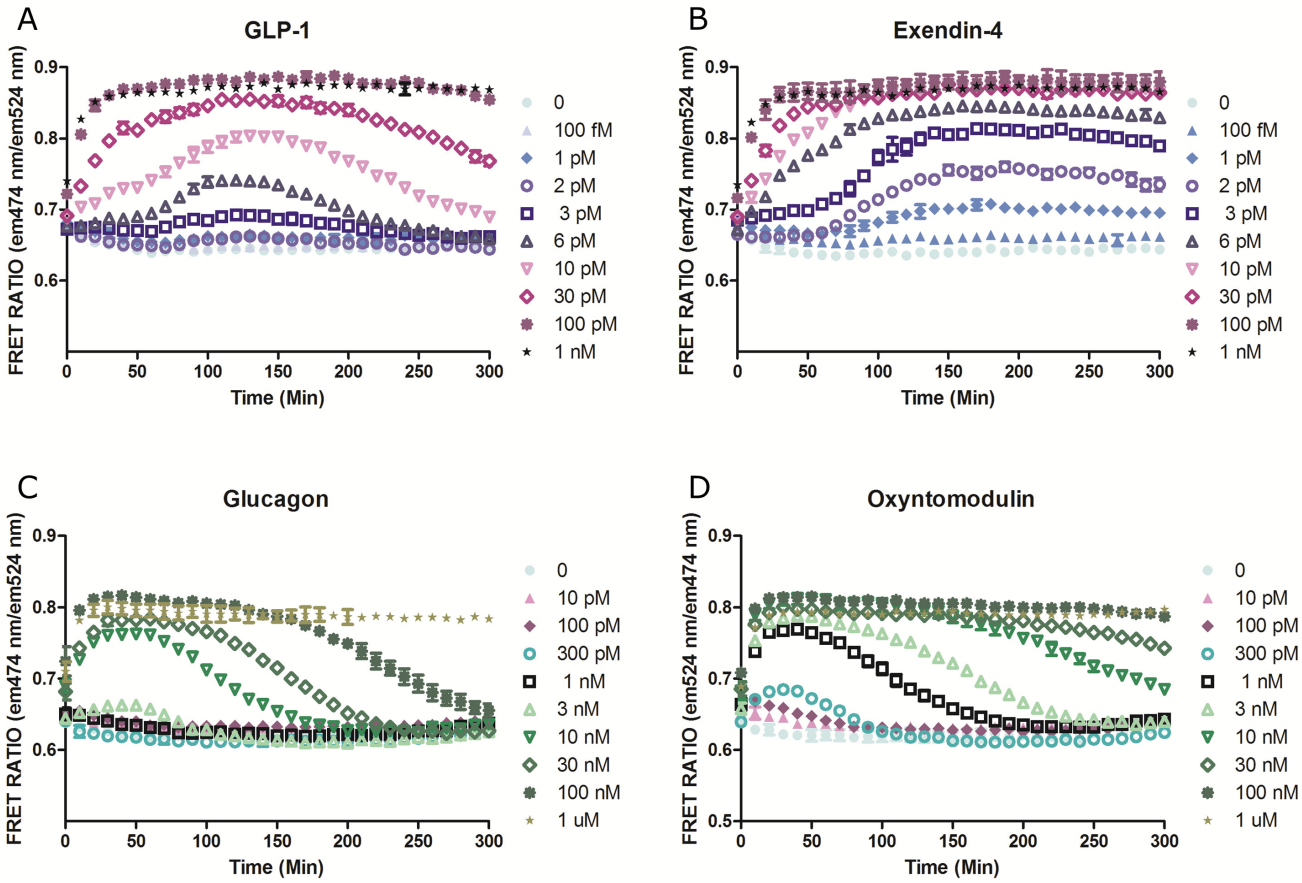


Figure 17. Agonist response kinetics in HEK293 cells expressing GLP-1R and Epac149. Varying concentrations of **(A)** GLP-1, **(B)** exendin-4, **(C)** glucagon and **(D)** oxyntomodulin elicit different cAMP profiles. Data represent one of three independent experiments.

The cAMP profiles illustrated in Figure 17 show that all four agonists stimulate cAMP production in a dose-dependent manner. The highest concentrations of GLP-1 (1 nM and 100 pM) appear to activate AC immediately, whereas lower concentrations of GLP-1 increase cAMP production slowly. After approximately 130 minutes, the cAMP levels peak followed by a slow decrease (Figure 17A). Similar observations were made for exendin-4, although exendin-4-stimulated cAMP levels decline more slowly compared to GLP-1 (Figure 17B). As expected, oxyntomodulin and glucagon activate the cAMP pathway with lower potency compared to GLP-1 and exendin-4. Interestingly, the cAMP profiles of glucagon and oxyntomodulin are very similar (Figure 17C and D, respectively), but different to those of GLP-1 and exendin-4. As for GLP-1 and exendin-4, the highest concentrations of glucagon and oxyntomodulin appear to activate AC immediately, but lower concentrations of the two peptides also cause a rapid increase in cAMP levels, which peak after approximately 40 minutes followed by a rapid decrease. In order to rule out that the decrease in cAMP levels observed for GLP-1, glucagon and oxyntomodulin was a result of peptide degradation, more ligand was added to the system after 130 or 40 minutes, respectively (Figure 18). Only ligand concentrations equal to the EC_{50} values of the ligands were studied.

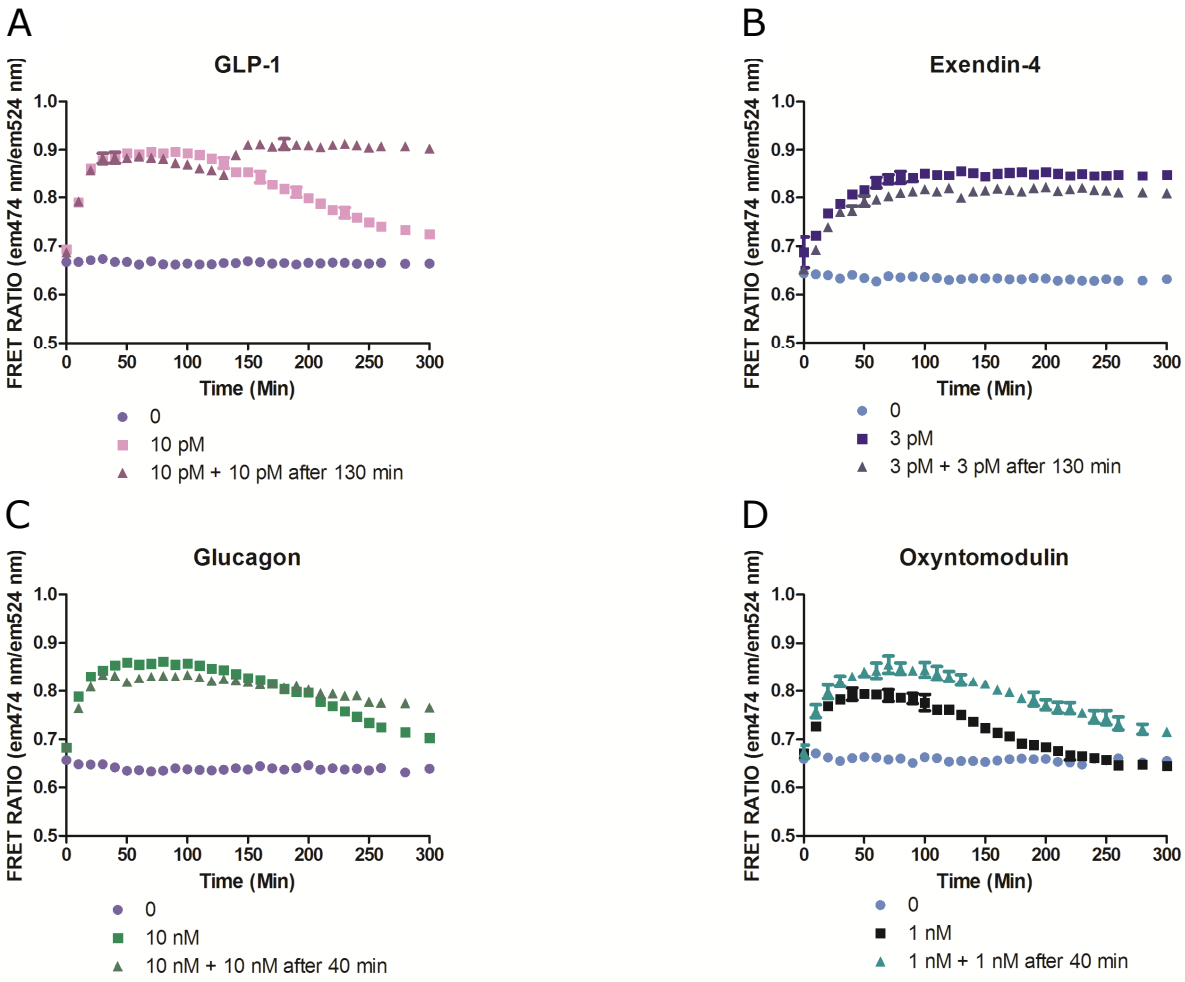


Figure 18. The effect of adding more ligand to GLP-1R expressing cells when cAMP levels start to decline. HEK293 cells expressing GLP-1R and Epac149 were stimulated with either **(A)** 10 pM GLP-1, **(B)** 3 pM exendin-4, **(C)** 10 nM glucagon or **(D)** 1 nM oxyntomodulin. Similar ligand concentrations were added to the cells after 130 minutes for GLP-1 and exendin-4, and 40 minutes for glucagon and oxyntomodulin. Data represent one of three independent experiments.

As shown in Figure 18, addition of more ligand has an effect on the cAMP production elicited by GLP-1, glucagon and oxyntomodulin, but not exendin-4. Hence, the cAMP profiles observed for GLP-1, glucagon and oxyntomodulin (Figure 17) may be affected by degradation of the ligand. The stability of GLP-1 and exendin-4 was subsequently examined in more detail using an in-house LOCI, which confirmed that GLP-1 but not exendin-4 is degraded over the course of the assay. As shown in Figure 19, the initial concentration of GLP-1 is reduced to approximately 30% after 300 minutes, whereas 90% of the initial concentration of exendin-4 remains after 300 minutes.

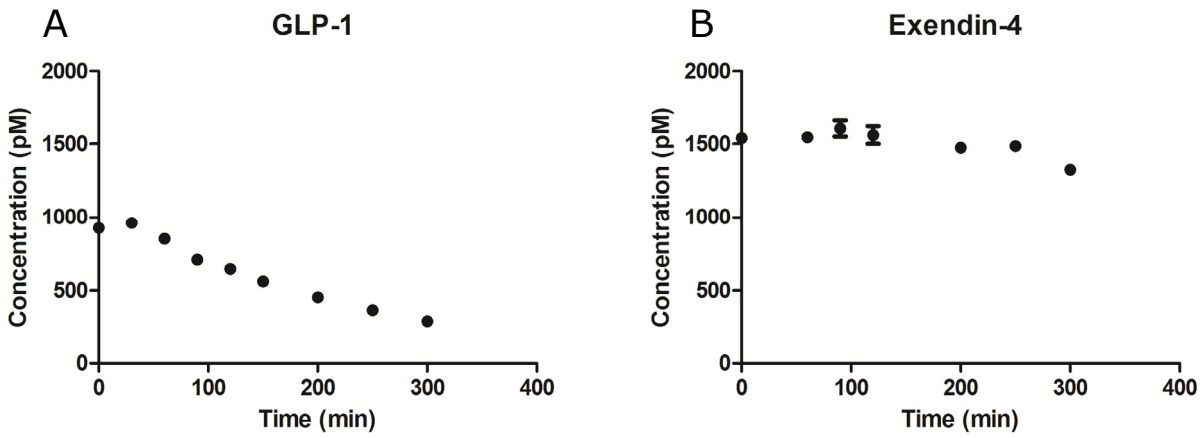


Figure 19. Stability of GLP-1 and exendin-4 over time. HEK293 cells transiently expressing GLP-1R and Epac149 were incubated with 1nM GLP-1 **(A)** or exendin-4 **(B)** for 0, 30, 60, 90, 120, 150, 200, 250 or 300 minutes. The resultant concentrations of each of the two ligands were quantified in an in-house LOCI.

In order to rule out the possibility that GLP-1 is cleaved by DPP-IV, I measured the cAMP profile of taspoglutide, which is a DPP-IV resistant GLP-1 analogue with similar potency and affinity for the human GLP-1R compared to GLP-1 (253). In addition, the cAMP profile of GLP-1 was measured in the presence of 10 µM ValPyr, which is a competitive DPP-IV inhibitor (254). The cAMP profile of taspoglutide was similar to that of GLP-1, and DPP-IV inhibition did not change the cAMP profile of GLP-1 (data not shown), which strongly suggests that the decrease in cAMP observed after 130 minutes (Figure 17A) was not a result of DPP-IV-mediated cleavage of GLP-1. However, other enzymes such as neutral endopeptidase (NEP) 24.11 may be responsible for the reduced stability of GLP-1 compared to exendin-4. NEP 24.11 is a membrane-bound metallopeptidase that cleaves GLP-1, exendin-4, glucagon and oxyntomodulin at multiple sites (57;255-257). The rate at which NEP 24.11 hydrolyses the peptides is as follows: Glucagon/oxyntomodulin > GLP-1 >> exendin-4 (256), which correlates well with the rapid decline in glucagon- and oxyntomodulin-induced cAMP levels and a delayed decline for GLP-1 and in particular exendin-4. It has been demonstrated that GLP-1 is degraded *in vitro* by NEP 24.11 present in plasma membranes derived from RINm5F cells (256), so GLP-1, glucagon and oxyntomodulin may also be degraded by NEP 24.11 present in HEK293 cell membranes.

Based on the results shown above, it seems likely that the cAMP profiles of GLP-1, exendin-4, glucagon and oxyntomodulin (Figure 17) reflect a difference in stability over time rather than different binding- and activation modes. However, I cannot rule out the possibility that the different cAMP kinetic profiles are a result of divergent effects on GLP-1R. One may argue that there is plenty of GLP-1 left after 300 minutes (the highest concentration ~ 300 pM) to elicit a cAMP response from GLP-1R (Figure 19A and Figure 17A). There is a striking similarity between the cAMP kinetics shown in Figure 17 and the potency rank order of the four peptides for GLP-1R (exendin-4 ≥ GLP-1 > oxyntomodulin > glucagon). Hence, the low potency agonists glucagon and oxyntomodulin cause a rapid increase and decrease in cAMP production, whereas the cAMP response induced by GLP-1 and in particular exendin-4 is slow, but sustained over several hours. It is tempting to speculate that there is a connection between the cAMP profiles and ligand binding modes and/or specific ligand-induced receptor conformations. Previous studies suggest that PTH receptor ligands with high affinity for a distinct receptor conformation elicit a prolonged cAMP response compared to ligands that

preferably stabilise other receptor conformations (258). A similar mechanism might explain why glucagon and oxyntomodulin elicit a rapid, but short-lived cAMP response, which is quite different from the prolonged response induced by GLP-1 and exendin-4. However, further investigations are required to support this. Differences in ligand-induced internalisation of GLP-1R could also affect the cAMP profiles observed in Figure 17. Hence, GLP-1 and exendin-4 may remain associated with GLP-1R and $G_{\alpha s}$ as the receptor internalises, whereas the low affinity of glucagon and oxyntomodulin could dissociate these ligands from GLP-1R upon receptor internalisation and in this way prevent prolonged signalling though $G_{\alpha s}$. It has recently been shown that $PTH_{1\text{-}34}$ remains associated with PTH-1R, $G_{\alpha s}$ and AC as the receptor is internalised, whereas PTH related peptide (PTHrP) does not (259). Interestingly, an Epac-based cAMP assay similar to the one used here, showed that $PTH_{1\text{-}34}$ elicited a prolonged cAMP response compared to PTHrP (259). This suggests that $PTH_{1\text{-}34}$-bound PTH-1R continues to signal through $G_{\alpha s}$ even after the receptor is internalised (259). Based on the cAMP profiles shown in Figure 17, it is tempting to speculate that similar internalisation patterns are observed for GLP-1R and high affinity ligands like GLP-1 and exendin-4. However, co-localisation of GLP-1R, GLP-1/exendin-4 and $G_{\alpha s}$ remains to be shown in subcellular compartments to support this hypothesis.

GLP-1R expression on the cell surface over time

In addition to studying the cAMP profiles of different GLP-1R agonists, I also used the real-time cAMP assay to study the presence of functional GLP-1Rs on the cell surface at different time points and different receptor expression levels using the specific GLP-1R antagonist exendin-4(9-39). HEK293 cells were transiently co-transfected with either 0.1, 0.5, 10 or 21 µg GLP-1R and 21 µg Epac149 DNA (per 15 cm dish), and the cAMP profile of GLP-1 was measured as described above. The presence of functional GLP-1Rs on the cell surface was confirmed by adding 1 µM exendin-4(9-39) after 5, 30 or 300 minutes. In Study I, Study II and Study III, 21 µg GLP-1R DNA was used for transfection, which resulted in high expression of the receptor. Conversely, transfection with 0.1 or 0.5 µg GLP-1R DNA is expected to cause low expression of the receptor. In this study, transfection with 0.1 µg GLP-1R DNA reduced to window of the FRET ratio dramatically (data not shown), whereas transfection with 0.5 µg GLP-1R DNA generated a FRET window comparable to that observed at high expression levels (Figure 20). As evident from Figure 20, the GLP-1-induced cAMP response was antagonised by 1 µM exendin-4(9-39) after 30 minutes at high as well as low expression levels of the receptor. This observation suggests that functional GLP-1Rs are present on the cell surface after 30 minutes of stimulation with GLP-1, and that this is not merely a result of receptor overexpression. Similar results were obtained when 1 µM exendin-4(9-39) was added 5 minutes after GLP-1 (data not shown). It has previously been shown that high concentrations of GLP-1 induce internalisation (measured as loss of surface receptor expression) of GLP-1R with maximal internalisation obtained after ~ 20 minutes (195). The presence of functional GLP-1Rs on the cell surface after 30 minutes indicates that GLP-1R may recycle back to the cell surface after internalisation. GLP-1R and other family B GPCRs such as GCGR and the PTH receptor have previously been shown to recycle back to the cell surface after internalisation (260-263). However, 1 nM GLP-1 may not be sufficient to induce receptor internalisation, so additional studies of the internalisation- and recycling patterns of GLP-1R in HEK293 cells are required to confirm this. It is worth noting that 1 µM exendin-4(9-39) antagonises the response of 1 nM GLP-1 when GLP-1R is expressed at low levels (Figure 20D), but not when GLP-1R is expressed at high levels (Figure 20B). Similar observations were made after transfection with 10 µg GLP-1R DNA (data not shown), which confirms that the high expression

level obtained with either 10 or 21 µg GLP-1R DNA leads to the presence of spare receptors on the cell surface.

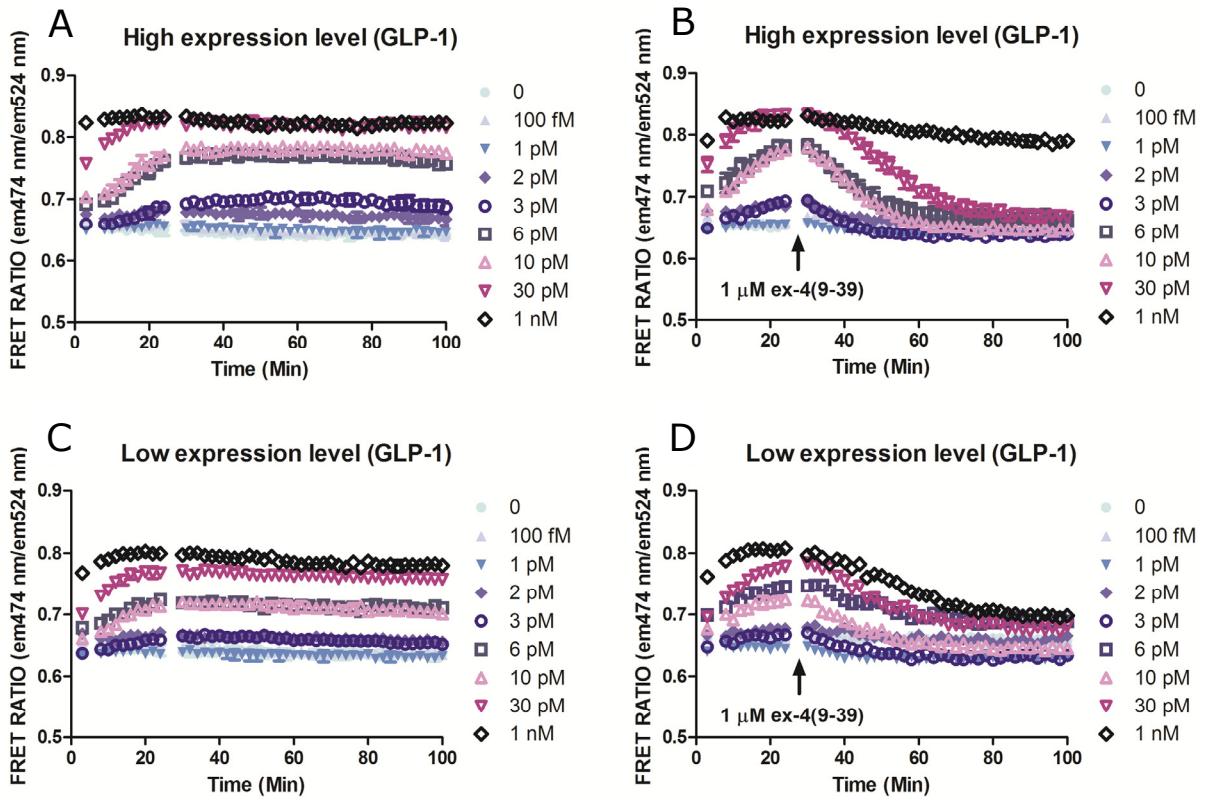


Figure 20. Agonist response kinetics in the absence or presence of a GLP-1R antagonist. HEK293 cells expressing high (21 µg DNA) **(A & B)** or low (0.5 µg DNA) **(C & D)** amounts of GLP-1R were stimulated with varying concentrations of GLP-1. Exendin-4(9-39) (ex-4(9-39)) was added after 30 minutes **(B & D)**. Data represent one of three independent experiments.

The cAMP profiles of GLP-1, exendin-4, glucagon and oxyntomodulin illustrated in Figure 17 represent the response of GLP-1Rs expressed at a high level, and cAMP levels were measured continuously for 300 minutes. In order to verify that functional GLP-1Rs were still present on the cell surface after stimulation with GLP-1 for 300 minutes, exendin-4(9-39) was added at this time point (Figure 21). Figure 21 shows that the GLP-1-induced cAMP response was indeed antagonised by 1 µM exendin-4(9-39), which strongly suggests that functional GLP-1Rs are present on the cell surface after stimulation with GLP-1 for 300 minutes. This may be a result of receptor overexpression, but it should be noted that 1 µM exendin-4(9-39) antagonises the response of 1 nM GLP-1 after 300 minutes (Figure 21B), but not after 30 minutes (Figure 20B). This could be a result of reduced surface expression of GLP-1R after 300 minutes compared to 30 minutes, or it may simply a result of GLP-1 degradation over time. Either way, functional GLP-1Rs appear to be present on the cell surface after 5 hours in this experimental setup.

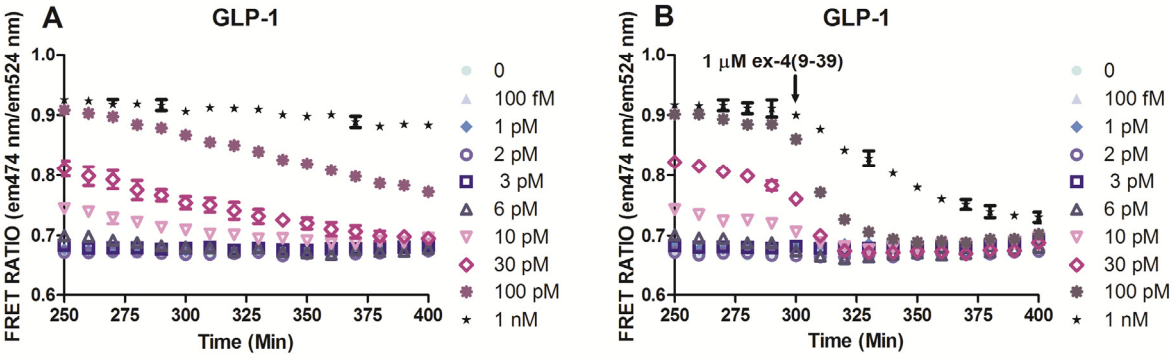


Figure 21. Functional GLP-1Rs on the cell surface after 5 hours of stimulation with GLP-1. HEK293 cells expressing high (21 µg DNA) amounts of GLP-1R and Epac149 were stimulated with varying concentrations of GLP-1 without **(A)** or with **(B)** addition of 1 µM exendin-4(9-39) (ex-4(9-39)). Exendin-4(9-39) was added after 300 minutes. Data represent one of three independent experiments.

Conclusion – Study IV describes the use of a FRET-based Epac biosensor to elucidate the cAMP kinetics of GLP-1R upon stimulation with high- and low potency receptor agonists. The results show a correlation between agonist potency and cAMP kinetics, as high potency agonists elicit a slow, but more sustained cAMP production compared to low potency agonists. Exendin-4 appears to have a prolonged effect on GLP-1R compared to other ligands. Whether this is a result of superior enzymatic stability of exendin-4, differences in binding- or activation modes or distinct ligand-induced internalisation patterns remains to be investigated.

CONCLUDING REMARKS

The overall purpose of this thesis was to investigate how GLP-1R interacts with receptor agonists. The hope was to gain valuable insight into receptor-ligand interactions and to identify domains in GLP-1R that are involved in receptor activation. Small molecule- as well as peptide agonists with high and low affinity for GLP-1R were included in the study, and a multidisciplinary approach was used to study receptor-agonist interactions. The work has contributed to a more detailed understanding of GLP-1R pharmacology in a number of ways:

- A crystal structure elucidated the molecular details of GLP-1 binding to GLP-1R ECD, and confirmed that GLP-1 adopts an α-helical conformation when bound to the ECD. In addition, the structure indicated that the α-helix of GLP-1 may be bent around Gly^{22} when GLP-1 is bound to the ECD of GLP-1R.
- A combination of crystallography and site-directed mutagenesis supported the existence of different binding modes of GLP-1 and exendin-4. In particular, the work demonstrated a ligand-specific effect of a Leu^{32}-Ala mutation in the ECD of the full-length GLP-1R. Whether the ligand-specific effect is affected by a kink in the α-helix of GLP-1 remains to be investigated.
- A cysteine-deprived and C-terminally truncated GLP-1R established that seven cysteine residues and more than half of the C-terminal tail are not required for GLP-1 binding or function.
- Real-time cAMP measurements indicated that exendin-4 has a prolonged effect on GLP-1R compared to other peptide agonists. However, additional studies are required to uncover the underlying mechanism of this effect.
- Receptor domains and specific residues involved in small molecule-mediated activation of GLP-1R were identified.

The crystal structure of GLP-1 in complex with the ECD of GLP-1R has not only clarified the molecular details of GLP-1 binding, it has also improved our understanding of the differential affinity of GLP-1 and exendin-4 for the N-terminal part of the receptor. After its release, the crystal structure has also been used in homology model structures of the full-length GLP-1R, which may in turn guide the rational design of improved therapeutic GLP-1-based analogues that target GLP-1R. The identification of receptor domains and residues important for small molecule-mediated activation of GLP-1R could also improve the design and development of small molecule GLP-1R agonists that are suitable for oral administration. It is obvious that rational drug design, regardless of whether it is applied to peptide- or small molecule ligands, would benefit immensely from a crystal structure of the full-length GLP-1R. The results obtained with the cysteine-deprived and C-terminally truncated GLP-1R may guide the design of stable receptor constructs that can ultimately lead to structural characterisation of the full-length GLP-1R.

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